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DEVOTED TO THE ADVANCEMENT AND DISSEMINATION OF KNOWLEDGE IN REGARD TO THE BACTERIA AND OTHER MICRO-ORGANISMS

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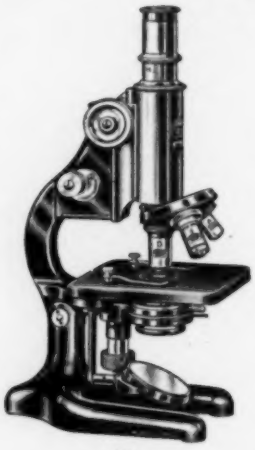

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OUR SOCIETY—IN RETROSPECT AND PROSPECT[1]

NORMAN MacL. HARRIS

Laboratory of Hygiene, Department of Health, Ottawa, Canada

From all the facts gathered concerning prehistoric man, the inferences show that he was a social animal. By force of many circumstances, physical and environmental, he was bound to be so in large degree, lest the forces of nature and his environment should have overwhelmed one so apparently unfitted to survive. It was in great measure the cultivation of his social instincts, I am convinced, that outweighed the adverse balance in nature against him and preserved the race.

With the passage of the thousands of years, this instinct has been preserved and accentuated. From its service as a purely protective measure, it has developed in a variety of ways, conserving customs, regulating habits, modes of living and of thought, and stimulating the growth, intercourse and exchange of ideas. In this latter quality of the primitive impulse as developed today, we find the cause of the grouping of civilized man in social circles under the guise of secret societies, benevolent societies, religious beliefs and scientific associations. In this latter group the old primitive force has found its widest expression, as witness the facts, that its bounds are neither sectarian nor national, but international, world-wide, giving the largest opportunity to mankind to reach its highest goal in aspirations, the full comprehension of truth and knowledge. In forwarding such aspirations, our Society is contributing its share.

To attempt a full résumé of our activities, past and present, is not the object of my address, but it might be well in this regard to make a few remarks for the benefit of our recent members, and

[1] Presidential address delivered at the twenty-seventh annual meeting of the Society of American Bacteriologists, Madison, Wisconsin, December 30, 1925.

to polish up the memories of the older ones, to show when and how the spell of the primitive urge manifested itself in our relations.

The Society of American Bacteriologists was founded in 1899, and held its first meeting at Yale University during the Christmas vacation of that year by the united action of some members still living and of others who have passed along. At the time it was felt that there should be some adequate channel of expression and interchange of ideas in the field of bacteriology, which had now in this country arrived at the definite status of a science, with the aim in mind to push forward its boundaries both in pure science and in the field of art and practice. The enthusiasm of those early days, the subjects discussed, debates on the papers and the social communion enjoyed, some of us can still recall. The uplift was felt by all, and pride in our organization was mighty. The years have passed and we still flourish, and our pride and enthusiasm, I take it, are not one whit diminished. From these small, but energetic beginnings of those early years our Society under the able guidance of such Presidents, as Sedgwick, Welch, Conn, Th. Smith, Novy, Jordan, Erwin Smith, Carroll, H. L. Russell, Kinyoun, Moore, Gorham, and of untiring, self-sacrificing secretaries as H. W. Conn, Marshall and Hitchens, our Society became solidly established, and has continued onward in the path of progress—a Society for which we today entertain feelings of the warmest regard and pride.

Touching now upon some of our outstanding achievements, your attention is directed to the following remarks:

One of the earliest ventures on our part, was the publication of the Society's "Descriptive Chart for the Identification of Bacterial Species," founded originally on previous efforts by the late Professor H. W. Conn and Dr. S. de M. Gage. This work was largely due to the efforts of Dr. F. D. Chester in 1903 to 1904, and a Committee on Methods for the Identification of Bacterial Species was formed with Dr. Chester as Chairman, his place being eventually taken by Professor Erwin F. Smith in 1907 when the chart was perfected and sponsored by the Society. In following years under various Chairmen, the chart underwent constant revision and has proved of wonderful assistance to all workers.

Out of the activities of this Committee, which was dissolved later on, grew the Committee on the Descriptive Chart, and in turn in 1920 a new Committee called the Committee on Bacteriological Technic was formed which had a broader field of activities accorded it and was placed under the able guidance of Dr. H. J. Conn who yet remains at the helm. This Committee has been responsible for the appearance of the admirable "Manual of Methods for Pure Culture Study of Bacteria" of which the Society is so justly proud.

Then in 1916 came the founding of the "Journal of Bacteriology" and in 1917 "Abstracts of Bacteriology," under the management of the Editors, Dr. C.-E. A. Winslow and Dr. A. P. Hitchens, respectively, and of an editorial board. Through the unceasing labors and skill of Drs. Winslow and Hitchens these publications have brought the Society international recognition.

Feeling the necessity of a more modern treatise on bacterial species than that useful book published by Dr. F. D. Chester in 1901, a Committee on Characterization and Classification of Bacteria was formed in 1917 under the Chairmanship of Professor D. H. Bergey, whose painstaking and highly commendatory efforts were published in book form in 1923, under the title of "Bergey's Manual of Determinative Bacteriology." In this field also the Committee on Taxonomy functioned under the excellent Chairmanship of Dr. C.-E. A. Winslow, and has published several papers by its members, in particular by Professor R. E. Buchanan, who under the aegis of the Society, has published this year the first volume of "Monographs on Systematic Bacteriology," which has received a very warm welcome throughout the English-speaking scientific world—and in turn, the reputation of the Society has been equally lustered.

In 1922 steps were taken to transfer the Type Culture Collection of Bacteria from the American Museum of Natural History, where it had been established by Dr. Winslow a few years previously, and to make it a part of our activities. This was accomplished and the Collection housed at the Army Medical Museum under the care of several of our indefatigable members. On account of unforeseen administrative difficulties it appeared

possible that the Society would have to abandon this one of its ventures, but a year ago, thanks to the generosity of the Director of the John McCormick Memorial Institute, of Chicago, Dr. Ludvig Hektoen, the Collection has found a permanent home under the efficient curatorship of Dr. Geo. H. Weaver.

Through one of our members, Dr. H. J. Conn, acting as Chairman, the Commission on Standardization of Biological Stains, sponsored by the National Research Council, has this year given us a splendid reference-book under the title of "Biological Stains," which will go far towards directing the attention of the new dye industry in this country to the needs of the biological sciences, and at the same time presenting all laboratory workers with a critical and comprehensive compendium which will give them a more intelligible knowledge of certain of the tools they work with. And, of course, you are already well aware of the very practical work this Commission is doing in certificating the validity and purity of biological stains made by American manufacturers.

But with the passing of time, it strikes me, in spite of our past accomplishments, that our marching step is not quite tuned up synchronously to the tramp of the legions of science in other spheres of knowledge. In this view I may be pessimistic, but with the passing of one's youthful enthusiasms, one is so prone to view present-day affairs in such a light, and I trust that you will pardon my croaking and bear with me.

Apropos of my remarks a moment ago, dealing with the early formative years of the Society, we must recognize that although we are only twenty-six years old, older perhaps than some of the members sitting before me tonight, we already possess a historic background and should take steps to preserve and add to it. So looking forward to the time when many of those intimately concerned in the initiative and struggling days of the Society will be passing off the stage, we should be up and doing, lest data easily available now be permanently lost to our records. Already, I am given to understand, documents of historic value are at this moment in other hands than those of the Secretary, where naturally they ought to be. It is believed that they are yet accessible, and every effort should be made to secure them.

All such material should without delay be collected, sifted and documented.

For an already overworked officer, this would prove to be impossible were he ever so willing, so to this end I offer the suggestion that there be permanently appointed, through proper channels, a member to take on the duties of Archivist or Historian, who, together with the Executive Officers and Council, would collect, evaluate and file all documents of possible historic importance, discarding all material reckoned to be worthless. This procedure might be repeated say every fifth year, in reference to further accumulations, and so there could be ensured a continuity of action throughout our existence, and give to further generations of members a glimpse of our progress from small beginnings. I can also conceive that our archivist might prove himself to be very human and entertaining if he were called upon to add to the zest of the Annual Dinner by giving, once in a while, brief discourses upon topics exhumed by him from the musty files under his care!

Our clothes of earlier years, having become too small for us, our Constitution underwent a much-needed revision in 1921, and again we are listening to complaints that we need a new suit. If this be so, by all means call in the tailor and trust that the subject of rehabilitation will be thorough and not long delayed. In this connection I would advocate the appointment by the President, of a standing Committee on Revision of the Constitution to whom members could forward suggestions which would then be dealt with on their merits and action taken through the usual channels.

In any contemplated revision of the by-laws, I would advocate the appointment of the Chairman of the Program Committee to be for a period of three years. Such a change would lead to a definite continuity of method in the framing of the annual program and would, I am confident, lead to the establishment of a permanently better balanced plan for annual meetings than at present prevails. Elsewhere, I have urged reform in methods of program planning, and perhaps a word or two might not be amiss here in this connection. I am convinced that provision should

be made in the program covering both the general meetings and each of the section meetings to enable the members to listen to one paper prepared, by request, by someone an authority on some topic of particular interest, followed by discussion prepared in advance by one or two members, then general discussion by anyone in the audience. Such a plan would lead to a thorough ventilation of the selected topic, and members in general would carry away with them a store of accepted opinion of the greatest value. Present day program planning such as we have pursued, makes no provision for a carefully prepared presentation of a subject of general or special interest.

Before leaving this subject of the program, another thought presents itself that might be given consideration, even though it may seem to tread upon the sensitive toes of custom. In many of our scientific societies at the present day, the opening number of the program is devoted to the President's address. This appeals to me very strongly as being the logical and appropriate time and place for it, rather than at the dinner where distractions of one sort or another tend to interfere with its presentation, and where at times the acoustic properties of the place sadly mar the pleasure of the listeners. To take the place of the presidential address at the dinner, I would suggest the organization of two or three brief orations by certain gifted members of the Society, or from one or two especially invited guests; such a departure, I anticipate, would in no wise detract from the pleasures of members accustomed to the old order.

With the steady growth of the Society in later years, its several activities have likewise expanded, so that it is plain to most of us that the time allotted to the business section of our program renders it now almost impossible to properly give the requisite degree of careful discussion to the multiplicity of business affairs, which constitutes so vital a part of the Society's fabric. To amend this state of affairs which is becoming each year more serious, I would advocate allotting one morning session to the business meeting, or even going the length of sacrificing a portion of that part of our program now given over to social functions. That such a move is radical, I do not deny, but the sub-

ject is one that is not to be denied and will sooner or later have to be faced by future executive officers.

In certain communities of men, as the result once more of the inherent primal social instinct, tradition often plays an important rôle in their development and progress. In some cases it proves highly beneficial to the community involved; in other instances it leads to cessation or retardation of progress; particularly is this so where tradition becomes a fetish, a thing to be worshipped, reverenced and hedged about. Tradition rightly evaluated is an object to be cherished, and though certain modes in our Society have been recognized and adhered to during the years of our existence, I scarcely think that we have yet earned the right to regard them in the light of anything traditional. So I venture again to offer a suggestion, which I feel, if tried out, would lead to giving added zest and interest to our annual gatherings. This would be the organization of what is known in some quarters as the "Round Table Conference." It is an informal gathering in the widest sense, and from personal knowledge of its operation I have formed the opinion that through its broadness of character, its freedom of expression, the wide range of topics touched upon in discussion, it would constitute a most valuable addition to our activities, particularly interesting to everyone, whether he listens in or actively participates. The meeting would be presided over by a chairman previously appointed in the general business meeting, who would with as little formality as possible, conduct proceedings. To cover as much ground as possible, ten minutes should be allotted to the speaker introducing his subject, and five minutes to those carrying on discussion. This "Round Table Conference" could readily enough take up one hour of the time usually set aside for the "smoker," thereby adding considerable profit to the entertainment.

I touch now upon another side of our activities, one which has caused all members considerable anxiety in the past and still exists to give us concern, namely, the question of keeping up our membership to such a level that cause for financial worry will cease. Some few years ago, under the very active chairmanship

of Mr. S. H. Ayers, an intensive campaign for membership was undertaken which was fruitful of immediate results, but was almost as soon counterbalanced by resignations in other instances, and today our numbers show only a slight advance. It is a discouraging situation, and one for which there does not appear to be an adequate solution at the present moment. I simply mention it here in the hope that each member will make up his or her mind by personal effort in any likely quarter to secure suitable additions to our membership. In connection with this question of membership, it seems to be an opportune moment to offer a suggestion which might go far to lessen the annual number of resignations. Let us consider that owing to our yearly change of meeting-places, we can at best count only upon an attendance of about 33 per cent of our total membership at any one assembly; of this number the percentage of regular attendants is by no means small. This then leaves a relatively high percentage of members who do not attend a meeting, say, once in four or five years. Amongst these loss of interest in the affairs of the Society insidiously creeps in, and this attitude of mind passes on into cold indifference, ultimately leading up to resignation. To keep up a vital interest amongst absentees, from whatever cause, I feel that the Society should make an effort to distribute among them a short report of its annual meeting. All that is necessary would be to publish as soon after the meeting as possible, a brief synopsis of the business session, together with any other items of interest apart therefrom. Let the bulletin be in any form so long as its contents can make such an appeal to the absent member that he feels that he is not losing touch with the Society and its affairs. The cost of printing and distribution cannot be very great, and the results accruing therefrom would, I am confident, justify the expense.

Directing our thoughts now into another channel, I cannot refrain from touching upon one of the phases of the late war, in as much as it holds forth a lesson for us. None of us who joined up, either as a "buck" private, an officer, or of whatever rank, failed to quickly perceive the great necessity and value of organization. Those of us who rendered special services on account of

our previous technical training were probably more impressed by this necessity for organization, sometimes of a most delicately balanced coöperation, where close liaison with persons in other services was demanded. I need not quote individual instances where undoubtedly the winning of the war in the last analysis, depended upon the results attained by such careful coöperation.

That such knowledge has since become widely diffused in civil life by those whose fortune it was to be called to the colors, is well known, and, what is more to the point, there is considerable evidence before us that such lessons gained in the armies have not passed out of mind unheeded, but have been and are being either advocated or applied in business, professional and scientific circles today. So I would now direct your attention to some examples and quotations dealing with the value of coöperative work in research, quite apart from any military reference and leading up to what I wish to advocate as adding possibly another to the several activities carried on by us.

To begin with, the brilliant discovery of insulin in 1921 to 1922 by Banting, Best and Macleod at the University of Toronto, constitutes an outstanding early example of success in research through the associated efforts of chemists, physiologists and clinicians. Its immediate success measured in the number of lives saved, and its tremendous stimulus given to group-research can hardly be imagined.

In the attempt to unravel the mysteries surrounding the activities of the human strain of the tubercle bacillus, a further splendid example of group organization in research is exemplified by the Research Committee of the National Tuberculosis Association under the direction of the Chairman, Dr. W. C. White of the United States Public Health Service at Washington. He has succeeded in enlisting workers in several of the seats of learning in this country to attack phases of the problems by specialists in bacteriology, chemistry, histology, pathology, clinical medicine and pharmacology. To my knowledge this constitutes the greatest modern group-effort in scientific research yet undertaken, and truly points the way for others to follow in.

Dr. Florence Sabin, now one of the Research staff of the

Rockefeller Institute, formerly a leading authority on research problems in the field of anatomy at the Johns Hopkins Medical School, in the course of her presidential address given before the American Association of Anatomists this year, speaking of some aspects of research, bearing upon my remarks, says:

"If you compare the titles of scientific articles in the journals of today with those of twenty-years ago, you will find that we are beginning to work in groups, often quite large groups. The nature of research is changing in such a way as to make this necessary." Continuing she cites the example, just mentioned, of the Research Committee of the National Tuberculosis Association, and the coöperation of chemists, histologists and clinicians in the recent and successful attack upon rickets.

A notable contribution to this theme has recently appeared in "Science" by Professor Chauncey D. Leake of this University, within whose walls we are meeting, wherein he demonstrates as an outcome of the necessities of war-time measures, the value of group research. The lessons so learned have resulted in the now well-known series of excellent studies upon the value of tryparsamide in the treatment of neuro-syphilis by himself, Professor Loevenhart and associates.

In stressing this modern movement, I do not for one moment want to be understood as advocating pushing into the background, the development and activities of the individual research worker. For from it, because it is so self-evident that the very successes to be gained through group-research depend so vitally upon combinations of persons of proven high attainment in research. But since science has become so increasingly complex, one may readily envision the fact that it is now beyond the powers of one individual to encompass some problems whose ultimate solution may lie scattered amongst a number of branches of science.

Without further elaboration of this subject, I beg to offer a proposal to the Society that as one of its future ventures in forwarding our science, it undertake at some early date the formation of a committee, which might be known as the "Research Conference Committee," whose duties it would be, first, to formulate some topics considered by it as worthy of intensive group-

effort; second, to stand ready and willing to receive and consider for action suggestions of the same sort submitted by any member; third, to invite certain well-qualified members to participate in the research; fourth, through executive channels to solicit financial aid, where necessary, from quarters where grants are already made available, or from new sources (see Bull. Nat. Res. Council, 1921, II, part I, No. 9); and fifth, to publish results of such research, as work done under the authorization of the Society. Such an endeavor in this newly developing method of investigation would tend to elevate our Society still further in the estimation of its fellows in science, and detract in no whit whatever from the reputation of those of its members, or others outside of the Society associated with them, in any coöperative venture into the realms of the present vasty unknown. Some may feel that research is a matter for individual effort alone, then to them my argument can carry no appeal, but surely, as I intimated before, there are extant many problems in bacteriology that today no one worker can ever hope to solve, but which await coöperative study. One comes to mind in regard to the phenomenon of bacteriophagy, which despite d'Herelle's brilliant researches and writings leaves many unconvinced as to the real cause and operation of so interesting a phenomenon. Under present conditions of individual research, the study of this phenomenon has led to discordant opinions regarding it, and years of scattered effort and argument may pass before an acceptable solution of it be established; studies in mutation offer another field for group effort; the etiology of influenza another, and so on.

Such as they are, these reflections of mine concerning the state of our "body politic" may, I hope, be acceptable of consideration. They are given in the conviction that our Society cannot remain in a static state, be that state ever so excellent. If we are thoroughly vitalized, then we must press forward towards newer accomplishments, and it is with such a point in view that my address, however unworthy, has been formulated; paraphrasing that so well-known phrase of Horace, I may say that "*tempora mutantur et nos mutandi summus in illis.*"

I cannot bring my words to a conclusion without expressing

to you all the warm feelings I entertain towards the members of the Society for having in the past year so highly honored me with their confidence in making me their chief executive officer; and in laying down the trappings of office, I beg to extend to all the other executive officers and Council my hearty thanks for their cordial coöperation with me, and particularly to that weary Atlas, our indefatigible Secretary, whose shoulders carry no light weight of constant responsibility—ours the glory, his the toil.

The annual report of the committee in charge of the American Type Culture Collection, shows that the available cultures have increased from 175 to 722 during the period February 1 to December 15, 1925, including 69 moulds and 122 yeasts. A total of 360 orders has been filled, involving the sending of 1540 cultures to 240 persons and institutions. The committee has established a price of one dollar per culture with addition for packing and postage. A temporary list of cultures is available. A printed catalogue is planned for the near future. Communications should be sent to The American Type Culture Collections, John McCormick Institute for Infectious Diseases, 637 S. Wood Street, Chicago, Illinois.

THE MAGNITUDE OF THE ERROR DUE TO AMMONIA AND ITS SALTS IN THE VAN SLYKE AMINO NITROGEN PROCEDURE AS COMMONLY APPLIED IN STUDIES OF BACTERIAL METABOLISM

L. B. PARSONS AND W. S. STURGES

From the Laboratory of the Cudahy Packing Company, Omaha

Received for publication, February 10, 1925

Although Van Slyke definitely states that the presence of ammonia may cause a serious error in the determination of amino nitrogen by his method, many bacteriologists have apparently failed to appreciate the importance of this point. He warns that,

> For the accurate determination of amino nitrogen in digesting solutions, etc., it is advisable first to remove ammonia although good comparative results can be obtained in the presence of the relatively small proportion of ammonia usually present, if the reaction conditions of time, concentration, and temperature of solutions are kept constant so that the proportion of the ammonia decomposed is the same in each determination.

He even presents some data (table 1) showing the amounts of nitrogen liberated from an ammonium sulfate solution when allowed to react for various lengths of time.

A brief survey of recent studies of nitrogen metabolism of bacteria (Wagner Dozier and Meyer, Avery and Cullen) failed to show that these investigators had made any allowance for ammonia interference in their Van Slyke amino nitrogen determinations. Others (De Bord and Lamson) have criticized and discarded the method without considering the importance of the ammonia interference.

Kendall recognized the indefiniteness of the ammonia correction and therefore gave preference to the Sörensen method

for amino nitrogen. Reddish and Rettger apparently recognized the ammonia error since they removed the ammonia before analyzing for amino nitrogen by the Van Slyke method.

In view of the conflicting experiences of various investigators, it seemed worth while to undertake a brief physico-chemical investigation of the nature of the reaction of ammonium salts in the Van Slyke procedure, and to determine the magnitude of the error under various conditions.

TABLE 1

Used 10 cc. portions of ammonium sulfate solutions containing 28.02 mgm. of nitrogen. Temperature 24. Pressure 752 mm.

TIME OF REACTION	NITROGEN	WEIGHT OF NITROGEN	PER CENT OF TOTAL AMMONIA NITROGEN
minutes	*cc.*	*mgm.*	
3	12.1	6.86	24.5
5	18.4	10.16	36.3
10	31.5	17.38	62.1

EXPERIMENTAL

The experiments detailed in this paper were carried out with the micro-apparatus. Reagents of a satisfactory degree of purity were used. The sodium nitrite solution used was made up to the recommended concentration of 300 grams per liter, unless otherwise specified. The ammonium salt solutions used were made from the purest materials available and the concentrations were verified by subsequent analysis for ammonia.

The quantities of acetic acid and sodium nitrite solutions were carefully measured in the apparatus and the volume in the reaction chamber was carefully adjusted to the 4 cc. graduation before admitting the sample.

The rapidity of shaking was adjusted to 250 to 300 r.p.m., this being deemed sufficient to remove all liberated nitrogen. The temperature of the room was carefully controlled and the reagents were kept adjusted to this temperature. The latter precaution is of the utmost importance as will be shown subsequently. The writers are satisfied that the temperatures recorded represent those of the reaction mixtures to $\pm 0.3°C$.

The details of the Van Slyke procedure are too well known to require further comment. The various periods of the reaction were timed with the greatest possible precision and the liberated

TABLE 2

The amount of reaction is independent of the ammonium salt used

Temperature 25°C. Pressure 735 mm. Shaking three minutes. Sample = 1 cc.

AMMONIUM SALT USED	CONCENTRATION (NITROGEN PER CUBIC CENTIMETER	NITROGEN EVOLVED	PER CENT TOTAL NITROGEN EVOLVED
	mgm.	mgm.	
Acetate	5.0	1.630	32.6
Butyrate	5.0	1.680	33.6
Chloride	5.0	1.679	33.6
Acetate	2.5	0.825	33.0
Butyrate	2.5	0.816	32.6
*Gelatin + butyrate	2.5	0.865	34.6

* Five per cent Nutrient gelatin (Difco) + ammonium butyrate (2.5 mgm. nitrogen per cubic centimeter) gave the following:

Average corr. cc. nitrogen from 1 cc. above	2.24
Average corr. cc. nitrogen from 1 cc. gelatin control	0.63
From butyrate	1.61 cc. = 0.865 mgm.

TABLE 3

Data to determine the order of the ammonia reaction

Temperature 25.5°C. Pressure 745 mm. Sample = 1 cc. Concentration = 5 mgm. nitrogen per 1 cc.

TIME	NITROGEN EVOLVED		PER CENT OF TOTAL NITROGEN EVOLVED
minutes	*corr. cc.*	*mgm.*	
2	2.05	1.02	20.4
3	3.10	1.52	30.4
4	4.10	2.13	42.6
5	4.70	2.46	49.2
8	5.90	3.13	62.6
10	6.30	3.36	67.2
15	7.80	4.15	80.3

gases were removed with the utmost celerity after the desired reaction time had elapsed. These details are necessary to obtain concordant results with ammonium salts, although such

precautions are obviously unnecessary with amino acids where the reaction proceeds to completion before three minutes (the usual time of shaking) has elapsed.

The factors which characterize any chemical reaction proceeding with a measurable velocity are: (1) the order of the reaction, (2) the temperature coefficient, (3) the concentration of the reactants.

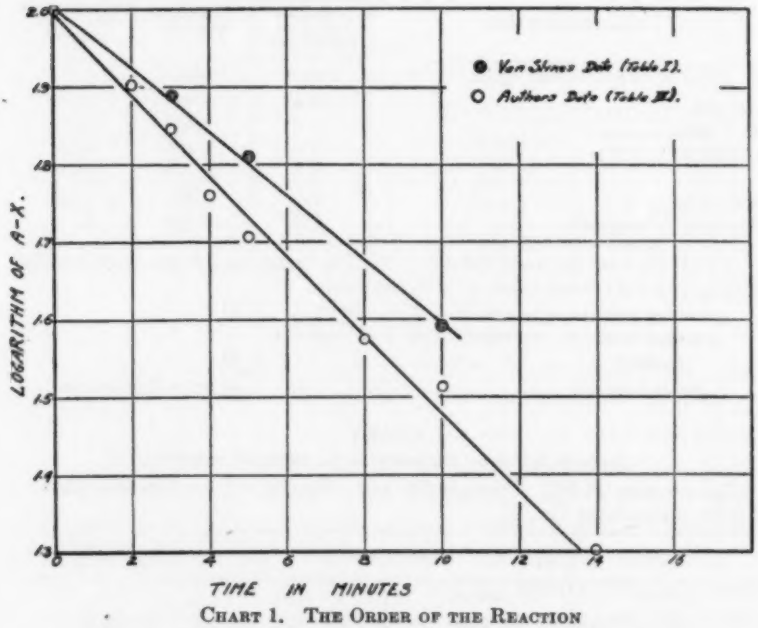

CHART 1. THE ORDER OF THE REACTION

It became necessary first to determine whether various ammonium salts reacted to the same degree. Table 2 shows the results obtained with a number of salt solutions.

It is evident from table 2 that the amount of nitrogen evolved is, within the limits of experimental error, independent of the salt used and is not influenced to any appreciable extent by the presence of even 5 per cent of gelatin.

The data given in table 3 were obtained with ammonium acetate to determine the order of the reaction.

If the reaction is monomolecular the logarithm of the concentration of the ammonium salt remaining in the reaction chamber at the end of time t plotted against time will be a straight line. The plots of the data in table 3 as well as the data of Van Slyke (table 1) are given in chart 1.

Both sets of results plotted in this way give straight lines. Their different positions on the chart are due to different reaction velocities resulting from different temperatures and different concentrations[1] of reagents in the reaction mixture.

TABLE 4

Calculation of velocity constant

t	x	$a-x$	$\frac{a}{a-x}$	$\log \frac{a}{a-x}$	$\frac{2.303}{t}$	k
			$a = 100$			
2	20.4	79.6	1.25	0.097	1.150	0.108
3	30.4	69.6	1.44	0.158	0.770	0.122
4	42.6	57.4	1.74	0.241	0.575	0.139
5	49.2	50.8	1.97	0.294	0.462	0.138
8	62.8	37.4	2.67	0.427	0.288	0.123
10	67.2	32.8	3.05	0.484	0.230	0.111
15	80.3	19.7	5.06	0.704	0.154	0.108
			Van Slyke's data (table 1)			
			$a = 28.02$			
3	24.5	75.5	1.32	0.121	0.770	0.0875
5	36.3	63.7	1.57	0.196	0.462	0.0905
10	62.1	37.9	2.64	0.422	0.230	0.0970

Table 4 shows the velocity constants (K) calculated from the monomolecular reaction law[2] for the data in tables 1 and 3. For the sake of uniformity the results are calculated in percentages.

[1] Van Slyke used the macro-apparatus with 10 cc. of sample. This corresponds to the concentration obtained if a 2 cc. sample is used in the micro-apparatus. The results in table 3 were obtained from 1 cc. samples.

[2] The velocity of a monomolecular reaction is given by the equation

$$k = \frac{1}{t} \log_e \frac{a}{a-x}$$

where k is the velocity constant; t is the interval of time during which the reaction takes place, a is the initial concentration and x is the change in concentration, i.e., the amount of nitrogen evolved in the time t. For further details consult any text book of physical chemistry.

The values thus obtained for k are probably as constant as could be expected when one considers the experimental difficulties involved.

Since the reaction is undoubtedly monomolecular it follows that the rate of reaction is independent of the concentration of the ammonium salt, i.e., the per cent of the total nitrogen evolved in a definite interval will be the same regardless of the initial concentration of the ammonium salt.

TABLE 5

The amount of nitrogen evolved is independent of the concentration of ammonium salt

t = 25°C. Sample = 1 cc. Shaking = three minutes.

CONCENTRATION (NITROGEN PER CUBIC CENTIMETER)	NITROGEN EVOLVED	
mgm.	*mgm.*	*per cent*
1.0	0.32	32.0
2.5	0.82	32.8
5.0	1.68	33.6

These results show that within the limits of the probable experimental error the above statement is correct and may be taken as additional evidence that the reaction follows the monomolecular law.

TABLE 6

Per cent of nitrogen evolved between 20°C. and 35°C.

Shaking = three minutes. Concentration = 5 mgm. per cubic centimeter. Sample = 1 cc.

AMMONIUM SALT USED	NUMBER OF DETERMINATIONS	TEMPERATURE	AVERAGE AMOUNT OF NITROGEN EVOLVED	
			mgm.	*per cent*
Acetate	3	22.5	1.255	25.1
	2	23.5	1.405	28.1
	2	25.5	1.565	31.3
	1	27.0	1.940	38.8
	1	29.5	2.140	42.8
	2	32.5	2.725	54.5
Chloride	1	22.5	1.410	28.2
	1	25.5	1.720	34.4
	1	27.0	1.920	38.4
	1	32.0	2.580	51.6

The rate of a chemical reaction is usually doubled or tripled with each 10° rise in temperature. The temperature coefficient for this reaction between 20°C. and 30°C. may be stated thus

$$C = \frac{k_{30}}{k_{20}}$$

k being the velocity constant at the specified temperature. For the actual determination of C it was considered that the most

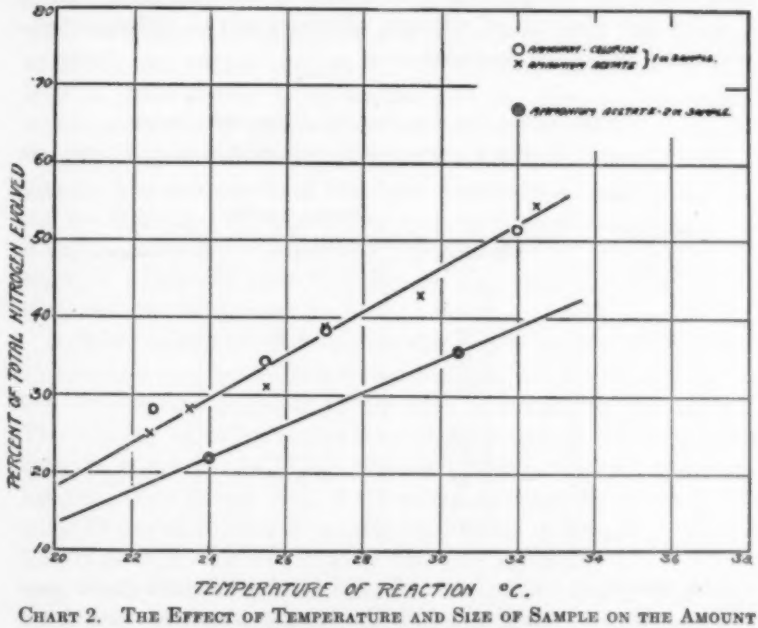

CHART 2. THE EFFECT OF TEMPERATURE AND SIZE OF SAMPLE ON THE AMOUNT OF AMMONIA NITROGEN EVOLVE

reliable result could be obtained by observing the amounts of nitrogen evolved in three minutes at various temperatures over this range.

Two sets of observations were made using two different ammonium salts. The results are recorded in table 6.

The results in table 6 are plotted in chart 2. The percentages of nitrogen evolved as taken from the curve at 20° and 30°C.

are 18 and 46 per cent respectively. This gives $k_{20} = 0.067$ and $k_{30} = 0.205$ and the temperature coefficient (20°C. – 30°C.).

$$C = 3.0$$

It is to be expected that the concentrations of the acetic acid and of the sodium nitrite will affect the reaction velocity. If this is the case, proportionate amounts of nitrogen will not be obtained from 1 cc. and 2 cc. samples. It is to be expected that the larger sample will give relatively less nitrogen. Table 7 shows the amounts of nitrogen obtained at two different temperatures from 2 cc. samples.

TABLE 7

Nitrogen evolved from 2 cc. samples of ammonium acetate

Sample = 2 cc. Shaking = three minutes. Concentration = 10 mgm. per 2 cc.

TEMPERATURE	NITROGEN EVOLVED		
	Milligrams	Average	Average per cent
°C.			
24.0	2.11	2.20	22.0
	2.27		
	2.22		
	2.22		
30.5	3.53	3.57	35.7
	3.57		
	3.50		
	3.65		
	3.60		

The values in table 7 are plotted in chart 2 and make possible a direct comparison with the results obtained with 1 cc. samples. The temperature coefficient (20°C. – 30°C.) calculated from this curve is 2.9 which is in good agreement with the value 3.0 obtained with 1 cc. samples. This speaks well for the consistency of the results.

DISCUSSION

It should be pointed out that the experimental results herein detailed are as close approximations as can be obtained with

the usual Van Slyke procedure. While greater precision could doubtless be obtained by introducing obvious refinements, it was rather the purpose of this work to observe the ammonium reaction under conditions usually employed.

The results show definitely that the reaction has a high temperature coefficient. This fact alone is sufficient to explain the failure of some investigators to obtain checks since in our experience the laboratory temperature may fluctuate 4° to 5°C. in a short period of time. Chart 2 shows that 18 per cent of the total ammonia is evolved in three minutes at 20°C., 32 per cent at 25°C. and 46 per cent at 30°C. Another factor which may lead to poor checks is failure to time the shaking accurately when ammonia is present. A smooth curve drawn from the data in Table 4 indicates that three and one-half minutes shaking yields 4 per cent more of the total ammonia than does a three minute shaking. This amounts to a very considerable error in many investigations, especially those studies of bacterial metabolism in which the ammonia nitrogen may be equal to or greater than the amino nitrogen.

Another factor which may give discordant results is the failure to measure accurately the reagents used, either initially, or in the deaminizing chamber at the time of admitting the sample. The effect of variation in the amount of sample is shown graphically in chart 2. In three minutes shaking at 25°C. the 2 cc. sample yields 24 per cent of its ammonia nitrogen as compared with 33 per cent for a 1 cc. sample. This difference is due to the dilution of the reagents by the larger sample.

The curves presented in chart 2 afford a possible basis for correcting results obtained from samples of which the ammonia content is known. The writers feel, however, that such a procedure is of questionable value since in most cases it is practicable to remove ammonia by some suitable method before making the Van Slyke determination.

The following results are offered to show what may be expected from a correction of Van Slyke analyses of material high in both amino and ammonia nitrogen. The sample used was a composite of several twenty-day-old nutrient gelatine cultures of *C. flabelliferum*.

	mgm.
Ammonia N (Folin)	345
Amino N (Van Slyke):	
1. NH_3 not removed, 1 cc. sample, 27°C	485
2. NH_3 removed by K_2CO_3 and aeration	334
3. NH_3 removed by $Ca(OH)_2$ and evaporation	371
Amino N (1) corrected[3] for NH_3	354

These results show that the corrected amino nitrogen is midway between the figures obtained after the removal of ammonia by two different methods commonly used. They further indi-

TABLE 8

ANALYSIS NUMBER	OBSERVER	CORR. CC.	NITROGEN PER 100 CC.	DEVIATION FROM MEAN	
			mgm.	mgm.	per cent
1	WSS	5.36	142.0	−1.1	0.77
2	WSS	5.41	143.3	+0.2	0.14
3	WSS	5.36	142.0	−1.1	0.77
4	LBP	5.41	143.3	+0.2	0.14
5	LBP	5.41	143.3	+0.2	0.14
6	LBP	5.46	144.6	+1.5	1.05
Mean			143.1		
1	LBP	1.96	51.9	+0.54	1.05
2	LBP	1.94	51.4	+0.04	0.08
3	LBP	1.91	50.6	−0.76	1.48
4	LBP	1.98	52.4	+1.04	2.03
5	LBP	1.95	51.6	+0.24	0.47
6	WSS	1.95	51.6	+0.24	0.47
7	WSS	1.93	51.1	−0.26	0.51
8	WSS	1.90	50.3	−1.06	2.06
9	WSS	1.92	50.8	−0.44	0.87
10	WSS	1.96	51.9	+0.54	1.05
Mean			51.36		

cate that a study should be made to determine the most suitable procedure for the removal of ammonia from bacterial cultures.

Despite the difficulties experienced by Lamson and others which have caused some to discard the method, the writer's

[3] At 27°C. (chart 2) 38 per cent of the total ammonia nitrogen is evolved. This amounts to 131 mg. giving a corrected amino nitrogen of 485 − 131 = 354 mgm.

results, after the removal of ammonia, have been entirely satisfactory. The results of two series of consecutive analyses on two different samples of twenty-day-old *C. sporogenes* cultures in nutrient gelatin are presented in table 8.

As may be seen in table 8 the maximum deviation from the mean in two series of analyses by two different analysts was 2.06 per cent. This error is almost certainly within the limits of the accuracy of the measurements of the 2 cc. sample. Lamson's maximum deviations from the mean as computed from his Tables II and III, which are comparable in amino nitrogen content to the latter part of table 8, are 7.2 and 9.4 per cent respectively. The cause for these greater deviations is presumably due to his failure to remove ammonia.

SUMMARY

1. Data have been presented to show; the order of reaction, the temperature coefficient and the effect of dilution in the ammonia decomposition in the Van Slyke amino nitrogen procedure.

2. It has also been demonstrated that by removing ammonia satisfactory checks can *consistently* be obtained for amino nitrogen.

3. Results obtained without the removal of ammonia are too high by 18 to 50 per cent of the total ammonia nitrogen present depending upon the temperature and the size of the sample used. This may readily cause amino nitrogen values to be 50 per cent too high in cases where the ammonia nitrogen is equal to or greater than the amino nitrogen.

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THE POSSIBILITIES OF THE CONDUCTIVITY METHOD AS APPLIED TO STUDIES OF BACTERIAL METABOLISM

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Wider application is constantly being made of physical methods by workers in the biological field. Such methods are often easy to apply and enable a large number of determinations to be made in a minimum of time. Still wider applications of such methods would doubtless be possible if adequate interpretations of the results were available. Electrical conductivity is a simple and precise determination which has proved a valuable tool in many branches of science but which has not found any wide application in studies of bacterial metabolism. Oker-Blom used the method in the study of fermentation by *B. coli* and *B. typhosus* but failed to reach any very definite conclusions. Sturges and Rettger used the method in a study of the proteolytic activity of bacterial enzymes.

The purpose of this paper is to present a resume of conductivity changes as correlated with ammonia and amino acid changes in cultures of *C. sporogenes* and *C. flabelliferum* in several media. These results were chiefly incidental to a comparative study of the nitrogen metabolism of these organisms.

EXPERIMENTAL

The conductivity equipment consisted of Leeds and Northrop instruments as follows: (*a*) No. 4760 Wheatstone bridge, dial type, direct reading, range 1–100,000 ohms; (*b*) No. 2370-B a. c. galvanometer; (*c*) No. 9816 transformer. This apparatus operates on a 110 V. a. c. circuit and has a guaranteed accuracy of 0.5 per cent. The conductivity cell, shown in figure 1, was

constructed by the senior writer and found entirely satisfactory. The details of construction are apparent. The stout copper leads h-h dip into mercury cups fixed above the thermostat and enable the cell to be quickly and easily removed for filling, cleaning etc. The capacity of this particular cell was 7 cc. The cell constant, using thrice recrystallized potassium chloride in N/50 solution was found to be 2.205. This was of a magnitude to insure resistance readings of a convenient size with the materials used.

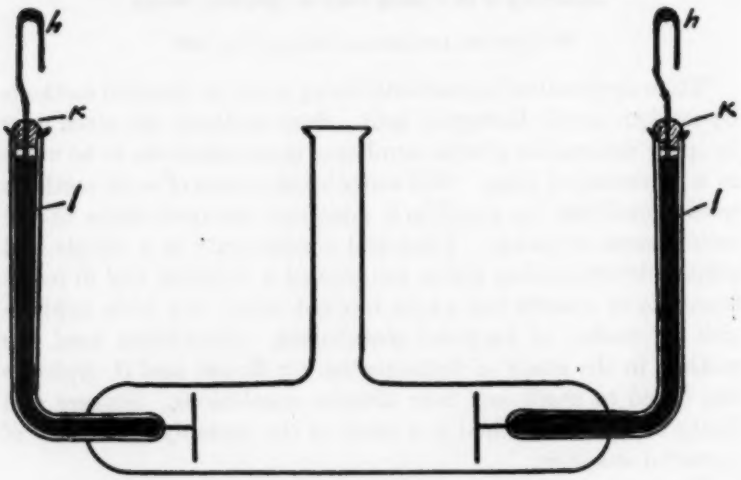

FIG. 1. THE CONDUCTIVITY CELL

hh, copper hooks; *kk*, DeKhotinsky cement seals; *ll*, mercury contacts to platinum electrodes.

The thermostat temperature was maintained at 30°C. during these determinations. It was found that thermal equilibrium was reached very quickly if the cultures were kept immersed in the thermostat water before filling the cell. Not more than five minutes was required for a determination, of the order of accuracy necessary in work of this character.

The media used were nutrient gelatin (Difco dehydrated), milk, and nutrient broth (pepton, 2 per cent). For each series five tubes of media were inoculated with each culture under

investigation. These were incubated at 35°C. in an atmosphere of hydrogen; and one tube was analyzed on the second, fourth, sixth, tenth, and twentieth day respectively. After the determination of the conductivity, the sample was used for the determination of ammonia (Folin), amino nitrogen (both Van Slyke and Sörensen (Brown, 1923) and electrometric pH.

After critically examining the large amount of data which became available in the course of these studies the writers concluded that a plot of conductivity change against change in ammonia and against the change in Sörensen values (amino N + ammonia N) would be most valuable in interpreting the results.

TABLE 1

Conductivity and nitrogen metabolism of C. flabelliferum in gelatin media

CULTURE	AGE	FORMOL TITRATION		AMMONIA NITROGEN		CONDUCTIVITY	
		Nitrogen per 100 cc.	Change	Nitrogen per 100 cc.	Change	Ohms^{-1} × 10^6	Change
	days	*mgm.*		*mgm.*			
Control	0	60		10		3.5	
C. flabelliferum, 1	2	143	83	60	50	6.2	2.7
	4	251	191	140	130	10.2	6.7
	6	286	226	179	169	11.8	8.3
	10	455	395	255	245	15.8	12.3
	20	792	732	424	414	23.6	20.1

In obtaining the values plotted, the initial values; i.e., the conductivity ammonia, etc., of the sterile media, were subtracted from each subsequent determination on the cultures.

Table 1 shows a typical series of results obtained with a 10 per cent nutrient gelatin culture of *C. flabelliferum*.

Chart 1 shows the data obtained on two series of determinations using two different strains each of *C. flabelliferum* and *C. sporogenes* in gelatin media. No differentiation has been made between the individual strains of *C. flabelliferum* and C. *sporogenes*, the former being designated by crosses and the latter by circles. There can be no question that change in ammonia is proportional to the change in conductivity over the wide range

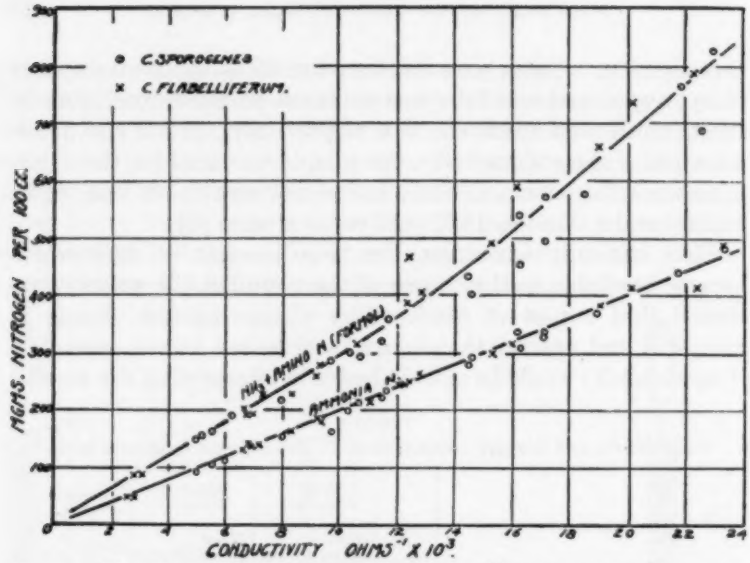

CHART 1. THE VARIATION IN AMMONIA AND FORMOL TITRATION WITH CHANGE IN CONDUCTIVITY IN GELATIN

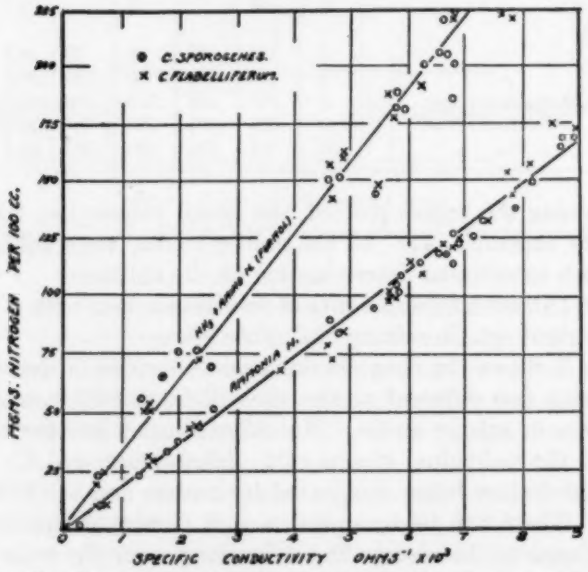

CHART 2. THE VARIATION IN AMMONIA AND FORMOL TITRATION WITH CHANGE IN CONDUCTIVITY IN MILK

studied. If the formol titration (the sum of amino and ammonia nitrogen) be taken as an index of proteolytic action, this chart further shows that change in conductivity represents fairly accurately the various degrees of proteolysis.

Chart 2 was plotted in a similar way from data obtained on three series of determinations using the same organisms in milk media. Here again it will be noted that change in conductivity

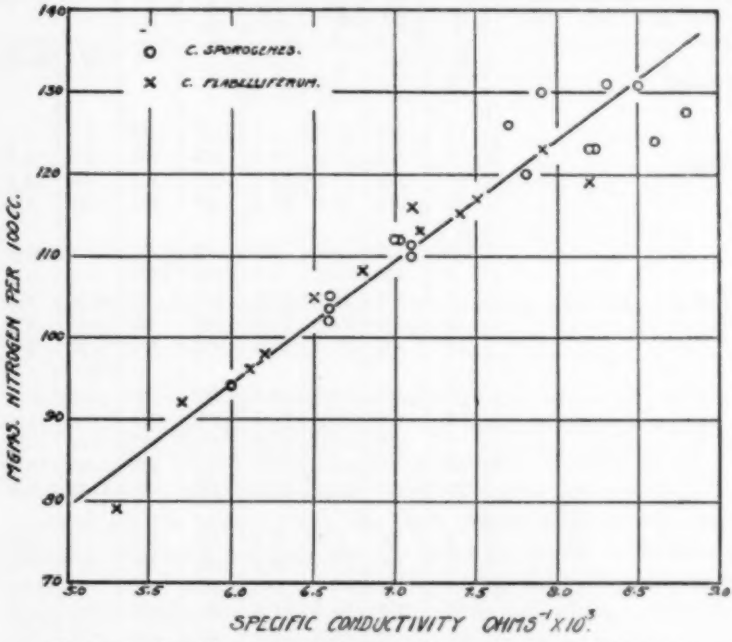

CHART 3. THE VARIATION IN AMMONIA NITROGEN WITH CHANGE IN CONDUCTIVITY IN PEPTON

is proportional to change in ammonia and also that change in degree of proteolysis follows change in conductivity.

The data on pepton media were not so satisfactory since a large amount of the total change occurred in the first two days. For the sake of completeness the ammonia data are presented in chart 3. It can be seen that here, also, the ammonia change is proportional to the change in conductivity. The formol values

TABLE 2

Comparison of ammonia nitrogen (Folin) with that calculated from change in specific conductivity

Eight strains of *C. sporogenes* in nutrient gelatin

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	OHMS^{-1} × 10^6 CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
Control	0	6	3.5					
W-66	4	163	11.6	8.1	157	158	+1	0.6
	6	161	11.8	8.3	155	162	+7	4.5
	10	283	17.4	13.9	177	171	+6	3.4
	20	413	23.7	20.2	407	394	−13	3.2
W-88	2	126	9.7	6.2	120	121	+1	0.8
	4	185	12.6	9.1	179	175	−4	2.2
	6	249	16.7	13.2	243	257	+14	5.8
	10	359	20.4	16.9	353	329	−24	6.8
	20	460	27.4	23.9	454	466	+12	2.6
W-84	2	138	10.4	6.9	132	134	+2	1.5
	4	226	15.0	11.5	220	224	+4	1.8
	6	256	16.9	13.4	250	261	+11	4.4
	10	350	20.1	16.6	344	324	−20	5.8
	20	460	24.5	21.0	454	409	−45	9.9
W-52	2	109	8.8	5.3	103	103	0	0.0
	4	188	12.8	9.3	182	181	−1	0.6
	6	198	13.1	9.6	192	187	−5	2.6
	10	317	18.4	14.9	311	291	−20	6.4
	20	440	24.0	20.5	434	400	−34	7.6
H-52	2	106	8.8	5.3	100	103	+3	3.0
	4	185	13.0	9.5	179	185	+6	3.3
	6	276	17.6	14.0	270	273	+3	1.1
	20	423	23.7	20.2	417	394	−23	5.5
H-54	2	168	12.0	8.5	162	165	+3	1.8
	4	223	15.2	11.7	217	228	+11	5.1
	6	227	15.1	11.6	221	226	+5	2.2
	10	290	17.6	14.2	284	277	−7	2.4
	20	424	22.5	19.0	418	371	−47	11.2

TABLE 2—*Continued*

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	OHMS^{-1} × 10^8 CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
H-48	2	132	10.2	6.7	126	130	+4	3.2
	4	165	12.5	9.0	159	175	+16	10.1
	6	193	13.5	10.0	187	195	+8	4.3
	10	286	17.4	13.9	280	271	−9	3.2
	20	436	23.7	20.2	430	394	−36	8.4
H-74	2	119	9.6	6.1	113	119	+6	5.8
	4	184	12.9	9.5	178	185	+7	3.9
	6	213	14.6	11.1	207	216	+9	4.3
	10	283	17.3	13.8	277	269	−8	2.9
	20	424	22.7	19.2	418	374	−44	10.5

are not shown on the chart since they were only a trifle larger than the ammonia values and could not be plotted without causing confusion.

Charts 1 to 3 show clearly that change in ammonia is proportional to change in conductivity. A further comparison of a large number of strains of *C. sporogenes* and *C. flabelliferum* on gelatin media, afforded an opportunity to give the conductivity method a rigid test. From the data presented in chart 1 it is possible to calculate a constant, for these particular organisms at least by which the change in specific conductivity must be multiplied in order to give the change in ammonia nitrogen in milligrams per 100 cc. This constant[1] is 1.95×10^4 for gelatin media.

Table 2 shows the results obtained for ammonia as calculated by the conductivity changes compared with the results obtained by the Folin method, using a number of strains of *C. sporogenes* in gelatin media.

[1] This is easily calculated by determining the slope in the equation of the curve in chart 1. The equation for a straight line is $y = mx + b$. Since the line pass through the origin $b = o$. Therefore, $m = y/x$ and it is only necessary to make a number of readings of x and y and perform the necessary division to obtain the constant.

TABLE 3

Comparison of ammonia nitrogen (Folin) with that calculated from change in specific conductivity

Eight strains of *C. flabelliferum* in nutrient gelatin

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	$OHM^{-1} \times 10^6$ CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
Control	0	11	3.5					
F-1	2	93	7.9	4.4	82	86	+4	4.9
	4	134	10.5	7.0	123	136	+13	10.5
	6	185	11.8	8.3	174	162	−12	6.9
	20	368	20.5	17.0	357	332	−25	7.0
F-2	2	161	11.6	8.1	150	158	+8	5.3
	4	213	14.7	11.2	202	218	+16	7.8
	6	299	17.0	13.5	288	263	−25	8.7
	10	363	21.2	17.7	352	345	−7	2.0
	20	444	24.9	21.4	433	418	−15	3.5
F-3	2	137	10.2	6.7	126	131	+5	4.0
	4	208	14.0	10.5	197	205	+8	4.1
	10	313	20.1	16.6	302	324	+22	7.3
	20	403	21.9	18.4	392	359	−33	6.7
F-4	2	244	14.1	10.6	233	207	−26	11.1
	4	287	17.7	14.2	276	276	0	0.0
	6	373	20.8	17.3	362	338	−24	6.7
	10	450	26.2	22.7	439	443	+4	0.9
	20	528	28.5	25.0	517	488	−29	5.6
F-5	2	140	10.3	6.8	129	133	+4	3.1
	4	196	13.3	9.8	185	191	+6	3.2
	10	267	21.4	17.9	356	350	−6	1.7
	20	423	23.4	19.9	412	388	−24	5.0
F-6	2	161	11.2	7.7	150	150	0	0.0
	4	223	14.3	10.8	212	211	−1	0.5
	6	447	23.7	20.2	436	394	−42	9.6
	10	382	21.8	18.3	371	357	−14	3.8
	20	442	23.8	20.3	431	393	−38	8.8

TABLE 3—*Continued*

CULTURE NUMBER	AGE	AMMONIA NITROGEN PER 100 CC.	SPECIFIC CONDUCTIVITY OBSERVED	$OHM^{-1} \times 10^4$ CHANGE	CHANGE IN AMMONIA		DIFFERENCE	
					Observed	Calculated		
	days	*mgm.*					*mgm.*	*per cent*
F-7	2	206	13.8	10.3	195	202	+7	3.6
	4	228	15.7	12.2	217	238	+21	9.7
	6	474	24.9	21.4	463	418	−45	9.7
	10	454	25.1	21.6	443	424	−19	4.3
	20	495	26.6	23.1	484	452	−32	6.6
F-8	2	175	11.9	8.4	164	164	0	0.0
	4	262	16.7	13.2	251	258	+7	2.8
	10	442	24.0	20.5	431	400	−31	7.2

Similar results with several strains of *C. flabelliferum* are given in table 3.

These results indicate that the factor derived from chart 1, using two strains each of *C. flabelliferum* and *C. sporogenes*, can be applied successfully for the calculation of ammonia changes from conductivity changes in gelatin cultures of many strains of these organisms. The maximum difference between the calculated and the observed ammonia change was 11 per cent and the difference was usually very much less than this. A close inspection of the tables shows that the higher ammonia values almost invariably show a lower calculated ammonia (from conductivity) than the observed value. This fact suggests that the ammonia-conductivity curve is not exactly a straight line but has a slight upward curvature such as is shown by the conductivity curves for pure ammonium salts in chart 4. If such is the case the constant already derived would not be exactly correct for all values. It would be possible to derive a more exact relation between conductivity and ammonia changes but it scarcely seems worth while at the present time.

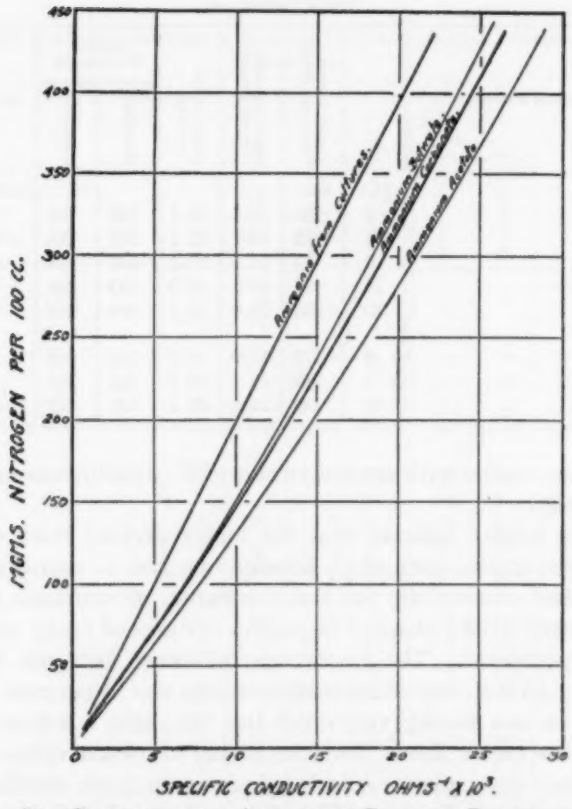

CHART 4. THE CONDUCTANCE OF AMMONIA IN CULTURES COMPARED WITH THE CONDUCTANCE OF VARIOUS AMMONIUM SALTS

DISCUSSION

The results herein presented seem to permit of the conclusion that conductivity may be used as a fairly exact measure of the ammonia produced by *C. sporogenes* and *C. flabelliferum* in several different media. It has further been demonstrated that the course of proteolysis as indicated by the formol titrations follows the change in conductivity rather closely. Since the ammonia nitrogen changes are so closely related to changes in specific

conductivity it became interesting to determine the specific conductivities of some ammonium salts which might well be present in gelatin cultures of these organisms. Measurements of the acetate, butyrate and carbonate at 30°C. are presented in chart 4. These are contrasted with the conductivity-ammonia changes observed in gelatin cultures of *C. flabelliferum* and *C. sporogenes*. These curves show that equivalent amounts of ammonium nitrogen give higher conductivities in the case of the ammonium salts than in the case of the gelatin cultures. There are many factors which have to be considered in attempting to explain this point. One obvious explanation would be that the treatment for the liberation of ammonia in the Folin method removes nitrogen which is not in the form of ammonium salts. Amide nitrogen might also conceivably be a source of this excess nitrogen. Again, ammonium salts of acids of lower conductivity than butyric might be present. Certainly it appears from the data in Chart IV that aspiration with sodium carbonate and oxalate is sufficient to remove all the ammonium in the form of ammonium salts. Any more drastic method proposed as being more efficient for an ammonia determination (Wagner, 1924) should be subjected to a very critical examination before being adopted. It might be expected that the nitrogen present in the form of amino acids, polypeptides and peptones would contribute materially to the conductivity. However, Bayliss in his study of the causes of conductivity changes in tryptic digests of gelatin presents data from which it may be concluded that the conductivity from these sources could not possibly account for much more than 5 per cent of the changes observed by the writers.

In any measurements of conductivity the pH change of the solution must be taken into consideration. The limits of pH in all the cultures studied were 5.5 and 8.0. It can be shown by a simple calculation that the specific conductivity due to the $(H+)$ at pH 5.5 is approximately 0.001×10^{-3} and that due to the (OH^-) at pH 8.0 is 0.0001×10^{-3}. These values are negligible in comparison with the observed changes.

An argument in favor of the conductivity method is the fact that in this measurement small changes in ammonia give large

changes in the resistance, and consequently in conductivity. For instance, when the ammonia nitrogen is increased by 400 mgm. the resistance in the cell used fell from 630 ohms to 110 ohms.

The writers recognize that the results presented may not be applicable to all cultures of proteolytic organisms. It has not been possible to undertake as extensive a study as would be necessary to establish the method on a satisfactory basis for a large number of organisms. There is no apparent reason why consistent results might not be obtained with any proteolytic culture. It is to be hoped that someone will have an opportunity to extend the scope of these observations, since the application of a simple method of this type can not fail to be of great value in studies of bacterial metabolism.

SUMMARY

1. The conductivity method has been suggested as a convenient means of following the rate of proteolysis in bacterial cultures.

2. It has been applied to a comparative study of several strains of *C. flabelliferum* and *C. sporogenes* in milk, nutrient gelatin, and pepton media.

3. The results establish the fact that in these media the change in conductivity is closely proportional to the change in ammonia, and follows rather closely the change in formol titration.

4. Data and calculations have been presented which show that the conductivity changes observed can be entirely accounted for by the ammonia found (Folin) if it is assumed to be present in the form of ammonium salts.

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FURTHER ISOLATIONS OF CLOSTRIDIUM FLABELLIFERUM WITH A COMPARISON OF THE NITROGEN METABOLISM OF SEVERAL STRAINS OF C. FLABELLIFERUM AND C. SPOROGENES

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A preliminary report has already been presented (1924) of an unusual type of putrefactive, anaerobic, clostridium. The most striking characteristic of this organism is the peculiar morphology of the sporangia, the vegetative portion of which invariably frays out longitudinally into innumerable fibers. This distinctive fan shaped appearance of the vegetative portion of the sporangium has suggested the name *C. flabelliferum*.

In the large number of isolations of putrefactive anaerobes made in this laboratory in the past four years, no organism similar to the culture originally isolated in 1921 was ever encountered until the summer of 1924. At this time a more or less routine examination of some materials from a western packing house revealed an abundance of anerobes having this striking morphology. This observation led to a limited bacteriological survey which disclosed the presence of putrefactive anaerobes of this type in salt (from Great Salt Lake), in sludge from salt leaching vats, and in certain specimens of some meats cured or partially cured with this salt. The dry salt in which a small amount of insoluble matter was present contained such a number of these putrefactive organisms that they were usually found in 1 cc. portions of a saturated solution and invariably in small portions of the flocculent sediment when inoculated into egg-meat media. The brush-like sporangia became visible after a few days incubation at 35°C. and in a few rare cases, where inoculation had been scanty, appeared to constitute a pure culture. Usually, however,

other organisms, both aerobic and anaerobic were present. In all cases extensive reisolations (in Veillon tubes, etc.) were resorted to. The absence of aerobes was, of course, assured by repeated failure to obtain growth aerobically on glucose agar slants using large inoculations. The absence of other anaerobes after repeated reisolations was established by exhaustive microscopic study. This is made possible by the property possessed by *C. flabelliferum* of retaining indefinitely in the spored state the frayed

FIG. 1. CLOSTRIDIUM FLABELLIFERUM—FOUR-DAY CULTURE IN EGG-MEAT MEDIUM MAGNIFICATION × 2000

vestigia of the vegetative cell. The presence of other anaerobic spores is readily detected among the characteristic sporangia of *C. flabelliferum*. *C. sporogenes* is especially easy to eliminate owing to its invariable early formation of free spores, which are never observed in pure cultures of *C. flabelliferum*.

Seven isolations of the new organism have been achieved which after repeated rigorous testing are considered to be pure cultures. These organisms have all been isolated from the materials mentioned above. In addition the presence of distinctive brush-

like sporangia has been noted in many other mixed cultures from the same sources.

Preliminary cultural tests wherein a comparison was made between two strains of *C. flabelliferum* (the original strain and a Salt Lake salt strain) and *C. sporogenes* (2 strains) has given some distinct differences. *C. flabelliferum* grown anaerobically in flasks of nutrient gelatin develops, sometimes within forty-eight hours, a few dense, viscid, gelatinous colonies adherent to the glass at the bottom of the flask. These colonies range from 1 to 4 mm. in diameter and have black centers with translucent borders. The turbidity of media offers another means of differentiation. *C. sporogenes* produces a heavy turbidity both in nutrient gelatin and in nutrient broth which persists even in old cultures. Cultures of *C. flabelliferum*, on the other hand, forty-eight hours or older, are invariably clear with the growth flocculated at the bottom of the tube. While the opportunity of observing the gelatin colonies has been limited to the first two strains, comparisons of turbidity have been made on all the cultures. This property was so striking that one could infallibly select the *C. flabelliferum* tubes from a collection of broth or gelatin cultures of the two organisms.

Since proteolysis is such an important consideration in the classification of anaerobes, it became of paramount importance to make a biochemical comparison of this property of the new organism with that of *C. sporogenes* which it closely resembles.

Various determinations have been proposed as quantitative criteria of proteolytic change. Among these may be noted ammonia, formol titration (amino + ammonia nitrogen), Van Slyke amino nitrogen, non-protein nitrogen, and the biuret test of Vernon. Considering the lack of agreement as to the relative merits of these determinations it appeared expedient to observe a number of these changes and to draw conclusions on the basis of all of the results.

As measures of proteolysis it was decided to follow changes in ammonia, formol titration, Van Slyke amino nitrogen, and conductivity.[1]

[1] The significance of conductivity change is discussed by the writers elsewhere.

By the proper choice of micro-methods it is possible to make all of these determinations in duplicate with 10 to 15 cc. of culture. This is a point of great convenience when so many observations are to be made using anaerobic procedure.

Ammonia was determined by a modified Folin-MacCallum method using 1 cc. samples with 1 cc. of a solution containing 10 per cent potassium carbonate and 15 per cent potassium oxalate. One cubic centimeter of kerosene prevented foaming. Ammonia was removed by aspiration into N/50 HCl and determined by titration of the excess acid using methyl red as an indicator.

Formol titrations were made on 1 cc. samples using J. H. Brown's (1923) modification B. of Sorensen's procedure. As an end point pH 8.0 was selected. Brown's precaution of titrating immediately after the addition of formol is to be emphasized for material of this nature in which CO_2 is undoubtedly present. For cultures in which ammonia and amino nitrogen is high, it becomes necessary to use a stronger sodium hydroxide solution (N/10 to N/5) for the final titration in order to avoid the dilution error. The writers consider that Brown's method is most satisfactory for use in bacteriological work. It was found possible to obtain checks to within 0.01 to 0.02 cc. consistently. A burette with 0.01 cc. graduations was, of course, used.

Van Slyke amino nitrogen determinations were made using the micro apparatus. Corrections for ammonia were made by the method suggested in another communication.

Conductivity was measured at 30°C. according to technique described by the writers in another paper.

The pH of all samples was determined electrometrically.

The rate of proteolysis was studied in nutrient gelatin (Bacto dehydrated), in beef extract broth (2 per cent Bacto Pepton), and in milk.

The cultures used were the eight isolations of *C. flabelliferum* previously mentioned (labelled F-1 to F-8) and nine strains of *C. sporogenes* obtained from the museum's collection and from Dr. I. Hall.

The media was tubed in 15 cc. portions and 0.2 cc. of a vigorous twenty-four-hour nutrient broth culture was used to inoculate

each tube. Media were always preheated to eliminate dissolved air. A sufficient number of tubes were inoculated with each

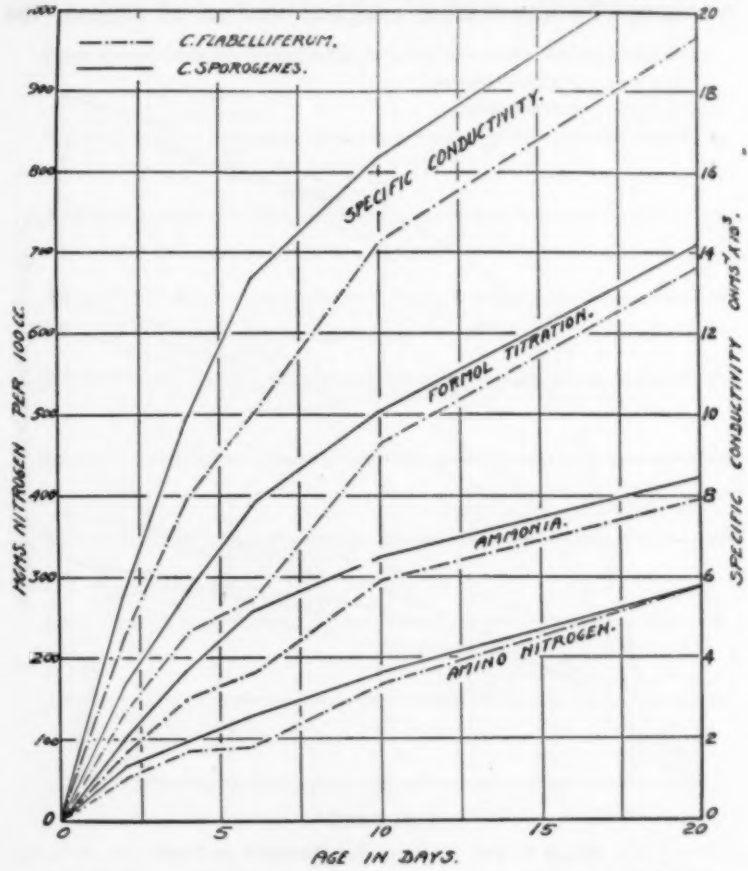

CHART 1. PROTEOLYTIC CHANGES IN NUTRIENT GELATIN MEDIA

Total nitrogen	1650 mgm. per 100 cc.
Formol nitrogen (initial)	60 mgm. per 100 cc.
Ammonia nitrogen (initial)	8 mgm. per 100 cc.
Specific conductivity (initial)	3.5×10^{-3} ohms.$^{-1}$

strain to furnish one tube for analysis on the second, fourth, sixth, tenth, and twentieth days respectively. Immediately

after inoculation the tubes were placed in suitable containers and anaerobic conditions were produced by evacuating and filling with hydrogen. To insure more complete removal of oxygen, the

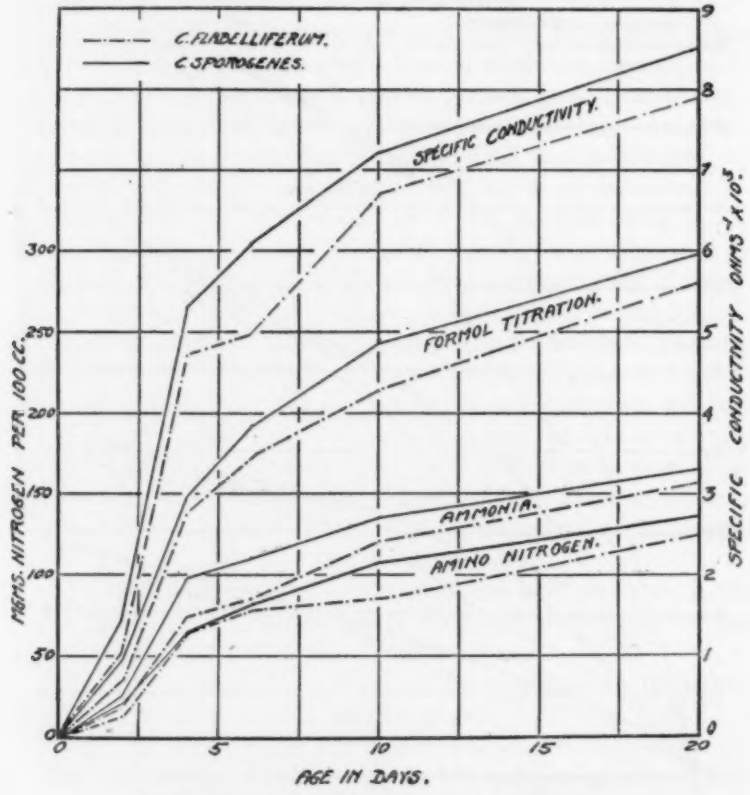

CHART 2. THE PROTEOLYTIC CHANGES IN MILK

Total nitrogen	515 mgm. per 100 cc.
Formol nitrogen (initial)	22 mgm. per 100 cc.
Ammonia nitrogen (initial	10 mgm. per 100 cc.
Specific conductivity (initial)	6.0×10^{-3} ohms.$^{-1}$

container was evacuated again and refilled with hydrogen. All cultures were incubated at 35°C. At the time of analysis each tube was tested for aerobic contamination.

Preliminary observations were made in three media (gelatin, milk and pepton) using two strains of each organism, designated as F-1, F-2; S-1 and H-52. Two or three series were run with

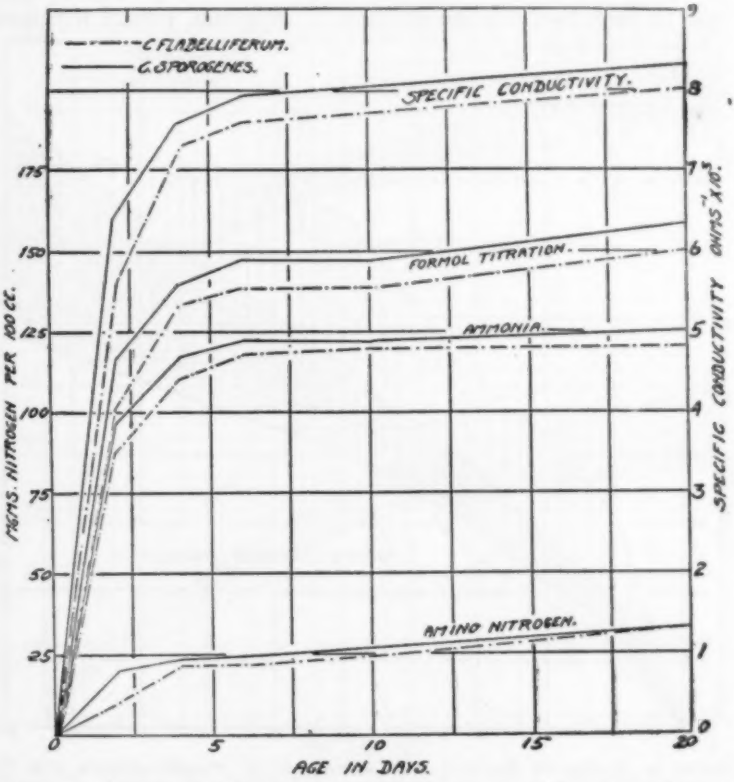

CHART 3. PROTEOLYTIC CHANGES IN NUTRIENT BROTH (2 PER CENT PEPTON)

Total nitrogen	319 mgm. per 100 cc.
Formol nitrogen (initial)	50 mgm. per 100 cc.
Ammonia nitrogen (initial)	10 mgm. per 100 cc.
Specific conductivity (initial)	5.0×10^{-3} ohms.$^{-1}$

ach media and the values obtained each day for the two strains f each organism were averaged. Such a mass of data results rom an extensive analytical study of this nature that it appears

neither necessary nor practicable to include the individual determinations. The writers consider that the material can be presented in the most intelligible form by graphical means. Charts 1, 2 and 3 summarize the results obtained. It will be noted that in each medium the changes in ammonia, formol titration,

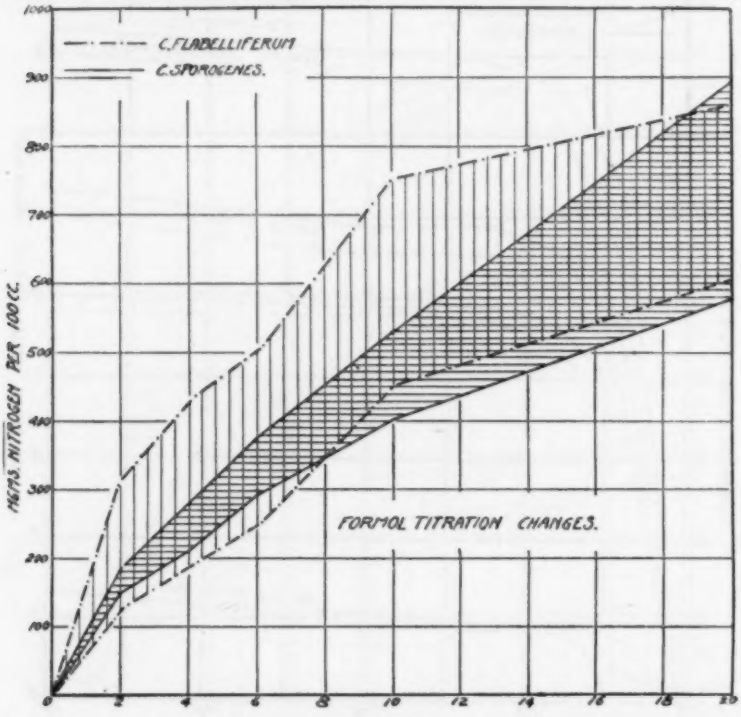

CHART 4. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES

Total nitrogen.....1650 mgm. per 100 cc.
Formol nitrogen (initial)..... 60 mgm. per 100 cc.

amino nitrogen (formol minus ammonia), and conductivity with time indicate that, at least for these strains, *C. sporogenes* is slightly the more proteolytic.

The general similarity of the time curves for the two organisms for each property measured necessitates the conclusion that the

nature of proteolysis of both anaerobes is essentially the same in any one of the media used.

Throughout the course of these observations it was apparent that there were consistent differences in the proteolytic activities

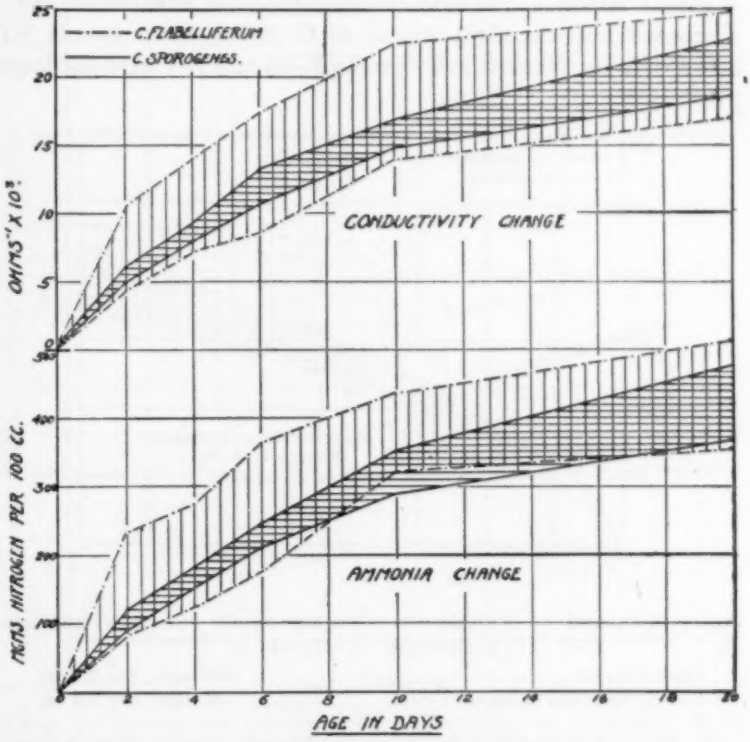

CHART 5. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES IN GELATIN

Total nitrogen	1650 mgm. per 100 cc.
Ammonia nitrogen (initial)	10 mgm. per 100 cc.
Specific conductivity (initial)	3.5×10^{-3} ohms.$^{-1}$

of the two strains of the same organism. Thus H-52 was the more active sporogenes and F-2 the more active flabelliferum strain. In the light of this observation the rather slight differences between the average curves of the two organisms becomes

of questionable significance. It, therefore, seemed advisable to determine the range of the proteolytic activities which may be exhibited by different strains of each of these organisms. Gelatin was selected as the most suitable medium for a comparative study. A careful survey of the data obtained from the nine strains of *C. sporogenes* and the eight strains of *C. flabelliferum* showed S-1 and F-1 to be the least active while W-88 and F-4 were the most proteolytic.

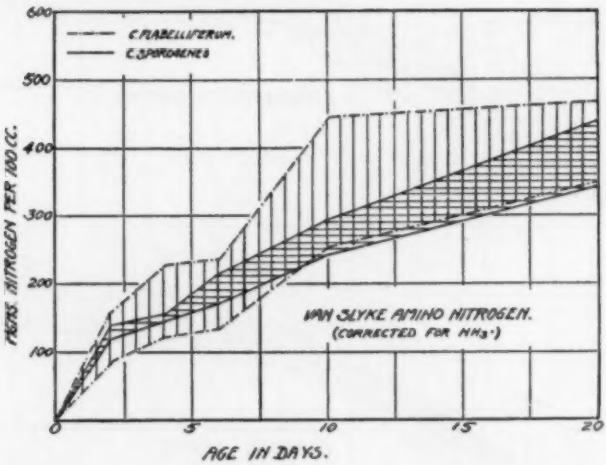

CHART 6. RANGE OF PROTEOLYTIC CHANGES BY C. FLABELLIFERUM AND C. SPOROGENES IN GELATIN

Total nitrogen	1650 mgm. per 100 cc.
Van Slyke amino nitrogen (initial)	63 mgm. per 100 cc.

In charts 4, 5 and 6 curves showing the changes in formol titration, ammonia, conductivity, and Van Slyke amino nitrogen with time for these four cultures are plotted. In all cases the lower solid line denotes culture S-1 which represents the least active sporogenes strain. The upper solid line denotes W-88, the most active sporogenes strain. The lower and upper broken lines denote F-1, and F-4; the least and the most active flabelliferum strains respectively. These charts show definitely that for the strains studied the zone of proteolysis of *C. sporogenes*

lies within the zone of proteolysis of *C. flabelliferum*.[2] The relationship of these zones suggests that, on the average, the flabelliferum cultures are more proteolytic than the sporogenes cultures.

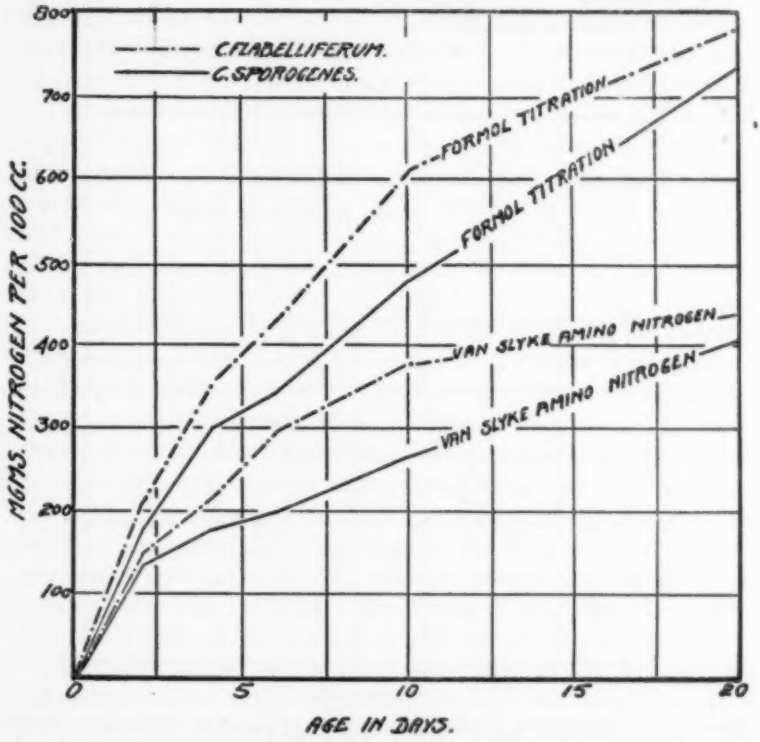

CHART 7. PROTEOLYTIC CHANGES PRODUCED IN GELATIN AVERAGING EIGHT STRAINS OF C. FLABELLIFERUM AND NINE STRAINS OF C. SPOROGENES

Total nitrogen	1650 mgm. per 100 cc.
Formol titration (initial)	60 mgm. per 100 cc.
Van Slyke amino nitrogen (initial)	63 mgm. per 100 cc.

Therefore, from the data average values of all the strains of each of the two organisms studied have been computed. These results are presented in charts 7 and 8, which show that at any

[2] *C. sporogenes* zone is represented by horizontal lining, *C. flabelliferum* by vertical lining while the zone in common is shown by cross lining.

age the average of the determinations of the change in any property is the greater in the case of *C. flabelliferum*. This

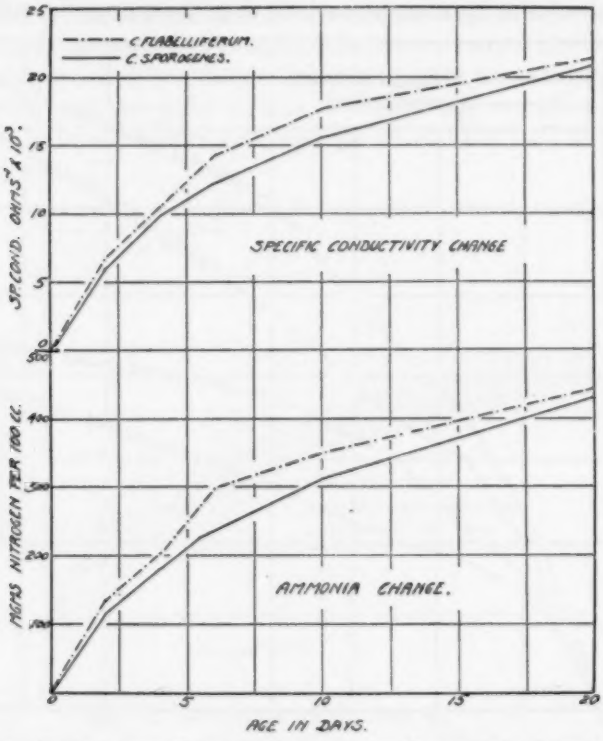

CHART 8. PROTEOLYTIC CHANGES PRODUCED IN GELATIN AVERAGING EIGHT STRAINS OF C. FLABELLIFERUM AND NINE STRAINS OF C. SPROGENES

Total nitrogen	1650 mgm. per 100 cc.
Ammonia, nitrogen (initial)	10 mgm. per 100 cc.
Specific conductivity (initial)	3.5×10^{-3} ohms.$^{-1}$

demonstrates that the distribution of the various strains throughout the plotted zones in charts 4, 5 and 6 is fairly uniform.

DISCUSSION

The results obtained in the preliminary studies carried on with two strains of each anaerobe would point to the conclusion that

C. sporogenes is the more actively proteolytic organism. However, results with a larger number of strains have demonstrated the opposite to be the case. The practice of attempting to make differentiations on the basis of quantitative differences is certainly to be deprecated unless it has been definitely shown that such differences are typical of a relatively large number of strains.

For these particular organisms it appears to be immaterial, which property is used to give a measure of the relative amount of proteolysis, since the charts show that in all cases the measurements of changes in conductivity, ammonia, formol titration, and amino nitrogen follow the same general course. The writers, of course recognize that while the above statement is true for these two organisms it may not hold for all proteolytic cultures.

Referring to charts 1, 2 and 3, it further appears immaterial which of these three media be employed in making a comparison of the proteolysis of these two organisms. The close agreement of the sporogenes curves with the flabelliferum curves for conductivity, for formol titration, for ammonia, and for amino nitrogen in any particular medium indicates that the nature of the proteolytic changes produced by the two organisms is essentially the same. The fact that in the three media the sporogenes average curves always slightly exceed the flabelliferum average curves is a striking confirmation of the foregoing conclusion and evidences the consistent behavior of the cultures.

Although differences between two given strains of the two organisms might easily be misinterpreted, the extensive data presented do not suggest any significant differences in the proteolytic activities of *C. flabelliferum* and *C. sporogenes*.

SUMMARY

1. Seven additional isolations of *C. flabelliferum* are reported which conform in distinguishing characteristics with the original.

2. An extensive comparison of proteolytic changes of the new organism with those of *C. sporogenes* is reported.

3. The nature of the proteolytic changes produced by the two organisms appears to be quite similar.

4. The critical survey of the data shows that differences in proteolytic activity between individual strains of the same culture may be more marked than differences between strains of *C. sporogenes* and *C. flabelliferum*. No definite quantitative differentiation between the two organisms is possible on the basis of this study.

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STUDIES IN MICROBIC HEREDITY

V. THE BIOGENETIC LAW OF HAECKEL AND THE ORIGIN OF HETEROGENEITY WITHIN PURE LINES OF BACTERIA[1]

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From the Department of Laboratories, Highland Hospital, Rochester, New York

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Many years ago the biologists, Haeckel and von Baer, made a most interesting generalization which stated that the life history of the individual of a species recapitulated the life history of the race—or in another form, that ontogeny recapitulates phylogeny. This law is still regarded by biologists as valid in a large way, although it cannot be successfully applied in detail, for the simple reason that entire phyletic stages may be suppressed or new non-phyletic adaptive stages interpolated, in a manner quite misleading to one who would unravel the phyletic or racial history of a species from its individual ontogeny.

The general method proposed by these investigators has been particularly fruitful, however, in our studies on the genetic relationships of bacteria. This method consisted, in brief, of making observations over a course of years on selected species (?) isolated from a single cell. For this purpose cultures to be suitable must exhibit a life history expanded into several clean-cut morphologic phases capable of ready reproduction. In other words there must be a clear delineation of the pleomorphic cycle. Figure 1 and diagram 1 show that our K A. diphtheroid strain answered fully this qualification. (See bottom of page 204 for explanation of plate 1.)

Figure 1 at *a*, for example, shows the "normal" or better the dominant phase, in the ontogeny of this culture. This is a rather slender, non-granular, Gram-negative bacillus. At *b* we

[1]Studies III and IV of this series appeared in the Journal of Immunology, Vol. XI, No. 2.

have a very coarse fungoid branching phase which gives rise to the gonidial and zygospore phases, *c* and *C*, and which is Gram-amphophilic. At *d* is a rudimentary branching phase not so coarse as *b*, and at *e* (diagram 1) is another bacillary phase which differs from *a* in being more retentive for the Gram stain. These represent the principal phases in the ontogeny of a pure line culture of this strain. To many bacteriologists it is still known only as the pleomorphic cycle and as such is without general biologic or medical significance.

Haeckel's Law suggests the possibility that these ontogenetic phases may stabilize under certain conditions as stages, variants or races—if one pleases—of this single organism. So stabilized they would then comprise its phylogeny. Indeed this result has been realized experimentally through the mechanism of the reorganization occurring in the sexual cycle. The first results with this strain were published several years ago (Mellon, 1920). The further experimental development of its phylogenetic history is the chief purpose of the present paper.

Previous work with the K. A. strain indicated clearly that under the conditions of the experiment it was possible to develop

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PLATE I

ONTOGENY

Fig. 1. K. A. 95.

Diagram 1. The small circle at the left hand side of the diagram indicates that this strain may be monophasic, i.e., it may multiply apparently to exclusion in the bacillary or so-called normal phase—*a* (Figure 4). The progressive widening of the circles to the right to include phases *e*, *d*, *b* and *c*, indicates that the culture may be bi-, tri- or tetraphasic, etc., and that each of these is capable of reverting to the original phase *a*.

PHYLOGENY

Fig. 3. K. A. 257 variant. Stabilized phase *d* of figure 1.
Fig. 4. K. A. 191 variant. Stabilized phase *e* of diagram 1.
Fig. 5. K. A. 105 variant. Stabilized phase *C* of figure 1.
Fig. 6. Fungoid phase *b*. Not stabilized.

Table of magnifications for photographs

× 1200: Figures 1, 2, 4, 6, 7, 10 and 11
× 1500: Figures 5, 20 and 21
× 2500: Figures 8, 9 and 19
× 3000: Figures 12, 13, 14, 15, 16 and 17

C d b

FIG. 1

DIAGRAM 1

FIG. 3

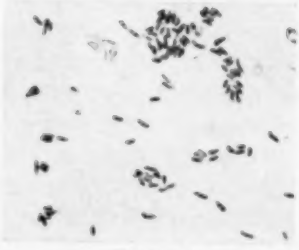

FIG. 4

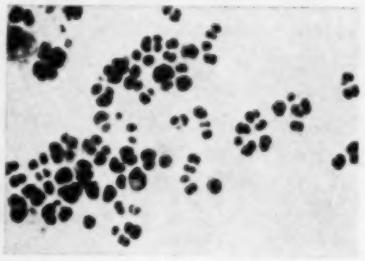

FIG. 5

FIG. 6

large numbers of what we spoke of as "giant coccoids," and which we now regard as probable zygospores (1920). The original culture of this pleomorphic strain (fig. 1) will be known as K. A. 95. It was further shown to be possible for these large forms to undergo a double segmentation and yield a true coccus mutant, which will be known in this study as K. A. 105 (fig. 5, plate I). It is now desired to show that the same or similar forms (fig. 2, plate II) will, under other conditions, give rise to a bacillary type of mutant (K. A. 257) (fig. 3, plate I); although the latter is closely related serologically to the antecedent bacillus, their cultural and morphologic characters are so different that one is surprised that this is the case.

EXPERIMENT I: THE MECHANISM OF MUTATION YIELDING STRAIN K. A. 257

The strain in question was isolated in single cell culture by Dr. M. A. Barber in 1919 and was planted in ordinary glycerol broth 0.5 per cent, having a pH of 6.8. As grown at 37° it produced many very large, rather poorly staining giant coccoid forms, originating from the coarse branching phase of the culture (fig. I, *b* and *C*). It was placed in the ice chest for about nine months which caused the broth to evaporate more than one-half. Owing to the lack of viability of other transplants of this strain, this culture was resorted to in an effort to rejuvenate it. At this time it had always grown on Loeffler's at 37° but transplantation under these conditions failed. Placed at room temperature for a week a luxuriant growth of a new type of bacillus was obtained on the Loeffler's slant. Microscopic examination of the old broth cultures showed that apparently no bacilli remained and that it consisted entirely of very deeply staining coccoid forms which varied greatly in size (fig. 2, plate II). The lack of the usual intracellular staining differentiations in these forms may possibly be correlated with the new type of bacillus evolved from them—a very different race from the coccus mutant referred to above.

The large numbers of colonies which germinated on the slant at room temperature left little doubt that the resulting bacillus germinated from these coccoid forms, particularly since none of

the original bacilli appeared. The origin of a new race from these coccoids was definitely proven for another strain of the diphtheria group (1922). The K. A. bacillus, as we originally obtained it, grew sparsely and was Gram-negative (phase *a*), while the large coccoid forms (phase *C*—zygospores) retained the Gram stain decidedly longer and could almost be called Gram-positive, while the variant bacillus which grew at room temperature was also very definitely Gram-positive as was the coccus mutant mentioned above.

Culturally the mutant bacillus, K. A. 257 (fig. 3) grew much more luxuriantly than the original strain, K. A. 95, and developed a buff colored pigment, while the original strain some years later

TABLE 1

	K. A. 257	K. A. 105	K. A. 95	K. A. 191
	pH	*pH*	*pH*	*pH*
Glucose	4.30	5.4	5.3	5.3
Mannitol	7.4	5.7	7.2	7.1
Levulose	4.29	5.6	5.2	5.3
Salicin	5.67	5.6	6.8	6.8
Dextrin	7.4	5.6	6.9	6.8
Inulin	7.4	6.6	7.0	7.0
Sucrose	4.44	5.6	5.7	5.6
Maltose	4.47	5.7	5.7	5.9
Lactose	7.6	6.4	6.9	6.9
Glycerol	7.6	6.8	7.0	7.0

developed a yellowish pigment. Furthermore, the variant strain was very different morphologically in that it showed a marked tendency to rudimentary branching forms, was quite granular, and culturally had a rather wrinkled, more cohesive character than the original bacillus, which was moist and shiny. These cultural differences were furthermore associated with the acquisition of the fermentation of salicin, as is seen in table 1, which gives the sugar reactions of the various strains.

EXPERIMENT II: THE ORIGIN OF STRAIN K. A. 191

Strain K. A. 191 (fig. 4) was derived from the pleomorphic K. A. 95 as follows: The pure line K. A. 95 had been kept on a blood

agar slant for some months in the ice chest. The growth was luxuriant but the pleomorphism slight, as was the rule on this medium. The rather fitful viability of this strain has already been mentioned, and it was notoriously weak on blood agar in contrast to a prolonged viability on Loeffler's medium where the gonidial or giant coccoid forms were usually numerous.

Most of the attempts to revive the blood agar strains failed whether transfers were made at 37° or 20° and regardless of the media tried; but for some unknown reason one tube which had failed to grow at 37° did finally grow at 20° on a blood agar slant. Morphologically the strain was identical, and it was at first thought that the original strain had been recovered; but the fact that it gradually developed a yellow pigment, even in young cultures, that it also grew luxuriantly on Loeffler's medium where its pleomorphism was greatly diminished, that it failed to develop numerous gonidia (giant coccoids) in serum broth, and that it was definitely—if not strongly—Gram-positive, made it clear that we were dealing with a variant.

Serologically it proved to be mutually reciprocal with K. A. 95, but the fact that it failed to form the large gonidia (zygospores) in appreciable numbers made it unsuitable for the coccus mutation experiments as originally described for it. Here is a fact of the *greatest importance in mutation experiments* because it may well account for the failure of one observer to repeat the work of another when he *fancies* he is working with the identical strain, merely because it is serologically identical.

When it is better appreciated that mutation is not apt to take place except through the medium of a special developmental stage of the culture, attention will be directed to developing an environment that will evoke such a stage. If this cannot be done, the culture is apt to remain very stable. Strain K. A. 191 is one of the few variants in which I could not be sure of the mechanism because the pleomorphic forms were not present in sufficient numbers to trace it readily.

Phylogenetically it is apparent that K. A. 191 (fig. 4) represents a stabilization of stage *e*) of the ontogenetic cycle (fig. 1 and diagram 1); K. A. 257 (fig. 3) represents stage *d*; K. A. 105 (fig.

5) represents stage *C*. The coarse fungoid stage *b* (fig. 6) has never been stabilized with this species, although as reported a number of years ago a true streptothrix stage of a fuso-spirillary complex was stabilized (Mellon, 1919a).

EXPERIMENT III: THE EFFECT OF SELECTIVE ENVIRONMENT IN THE DEVELOPMENT OF THE PLEOMORPHIC PHASES OF THE K. A. STRAIN

Of a wholly different order from this *permanent* stabilization of morphologic type, is the *temporary* reproduction that can be induced in these ontogenetic (pleomorphic) phases by a selective environment. Yet the latter is of importance if for no other reason than as conclusive proof that these pleomorphic forms are capable of multiplication. In such event they cannot properly be viewed as involution forms.

For example the rudimentary branching stage *d* in the original culture will reproduce *as such* to a limited extent if the pleomorphic mixture is transplanted to tall tubes of glucose ascitic broth where the conditions are largely anaerobic. Stage *a*, which is the normal bacillary stage, will not reproduce noticeably under these conditions. Transfer of these forms to the original conditions brings a return of the original picture. *No stabilization has occurred.*

We have frequently observed, too, the temporary reproduction of phases *b* and *C* under the following conditions. These phases developed readily on Loeffler's slants at 37° and seemed directly connected with the prolonged viability of the organisms, since old cultures on media which did not yield phase *C* were usually non-viable.

Even in old cultures on the Loeffler's medium the bacillary phase *a* was often non-viable, and on transplant to fresh media phases *b* and *C* often grew temporarily to the practical exclusion of the bacillary phase, as seen in figures 7 and 8. However, after twenty-four hours phase *b* began to dissociate the normal bacillary phase *a*, which thereafter completely dominated the picture. This reproduction could be observed directly under the

warm stage much after the manner of germination observed by Gardner (1925) with the "Y" forms of the dysentery group.

It should be perfectly clear then that any one ontogenetic phase of a culture may multiply almost to the exclusion of the other phases under conditions suitable for it, without mutation having occurred; but it should be equally clear if the multiplication is to be continued under a variety of conditions that the reorganizations incident to the sexual cycle must condition it, as indicated in the genetic origin of the mutant strains, K. A. 105 and K. A. 257.

Inasmuch as the K. A. strain is a diphtheroid it is of great interest to summarize briefly the work of others with the well known Park No. 8 diphtheria strain, since their collective observations point definitely to the existence of an ontogenetic cycle for this strain. Many years ago Dr. Anna Williams (1910) threw suspicion on the involutionary nature of the "branching involution forms," so-called, of this strain when she showed by direct observation that their metachromatic granules seemed to fuse before fission took place, and this fission she interpreted as a primitive sexual process. Moreover these branching forms were the only ones in the culture to show active growth and division under the condition of the experiment.

Just recently Martin, Loiseau and Gidon (1924) have noted that the same strain under partially anaerobic conditions yielded long filaments with lateral branches. Although this development begins after twenty-four hours and reaches its maximum by the eleventh or thirteenth day, the forms are still strongly Gram-positive, even after sixty days at 37°. It is significant that they consider these forms *not as evidence of degeneration*, but as normal developments under partially anaerobic conditions.

Again Heinemann (1917) working with this same strain noted that its almost complete loss of toxin producing power was correlated with a radically changed morphology. He found it to be reproducing in streptococcus and diplococcus form which bred true on glucose agar. Transplanted to Loeffler's medium the bacillary form of normal virulence was restored. It is obvious that this Park No. 8 strain undergoes important biologic alterations which are associated with its ontogenetic or pleomorphic

cycle and as such is strictly comparable to our K. A. strain, further evidence of whose heterogeneity will now be considered.

THE ORIGIN OF HETEROGENEITY IN BACTERIAL CULTURES

These experiments indicate clearly that the pleomorphic forms of a bacterial culture are *in a sense* independent entities capable of multiplication *as such* for a limited number of generations, at least. If—as will be shown—these phases have other physiological differences which emphasize their individuality, then we are forced to the conclusion that such cultures are in reality heterogeneous assemblages of individuals whose origin is the pleomorphic cycle.

In addition to the fact that these individual phases may reproduce as such, it is important to note that the mechanism of reproduction is not always the same. Phases *a* and *e* reproduce by transverse fission; phase *b* by branching, gonidial and zygospore formation (fig. 1, 6 and 7); and phase *C* by budding (figs. 8 and 9 at *a*). The latter being an ontogenetic phase, it proved impossible of course to isolate it, yet multiplication by budding could be observed directly under the microscope. Moreover the inoculation material contained relatively few of these circular forms (cf. fig. 1); yet in the transplant they dominated the morphologic picture (fig. 8) until they were overgrown later by the normal bacillary phase. The latter originated from the coarse branching phase *b* of figure 7. Here is definite proof of *reproductive heterogeneity* in a pure line culture.

The increase viability of phases *b* and *c* indicate cultural heterogeneity in respect to this character. As indicated, the origin of phase *a* from *b* and *c* prevented the cultures from dying out altogether. Old cultures of the original strain which did not contain these phases stained poorly, if at all. This was the case when the strain was grown on plain or blood agar instead of Loeffler's; but on the latter medium where the cultures were viable for many months, or even for more than a year, phases *b* or *c* were the only staining forms in the culture. Variant strain K. A. 191, representing phase *e*, maintains viability with little or no reproduction of the special phases on ordinary media or Loeffler's;

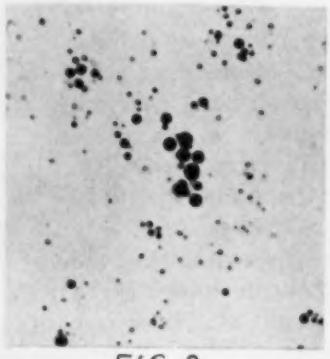

FIG. 2

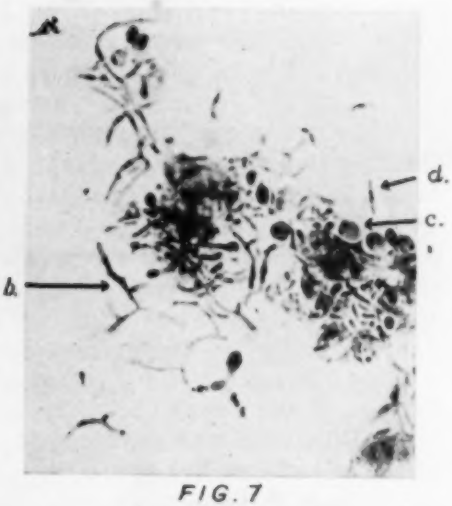

FIG. 7

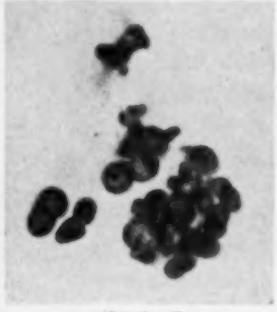

FIG. 8

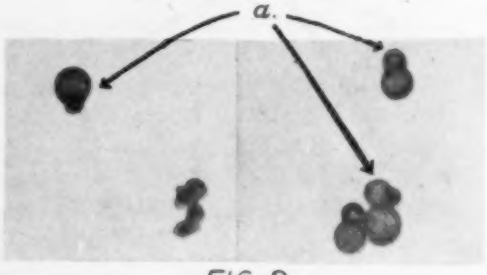

FIG. 9

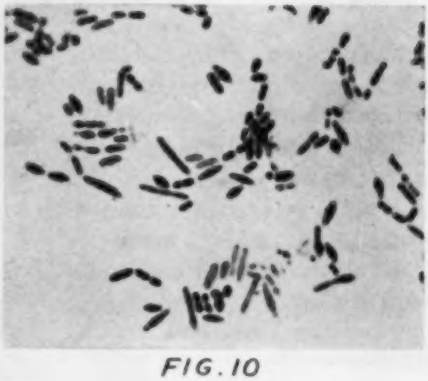

FIG. 10

FIG. 11

yet on egg media these organisms frequently appear in fair numbers, showing that the capatiy for their formation had been suppressed but not entirely lost.

The variant strain K. A. 191 had acquired pigment formation and grew readily at 20°, which was not the case with the original strain. This temperature adaptation was suggestively associated with the gonidial phase *c*, since when these forms were present in an old culture it germinated readily at 37° but not at all at 20°, while old cultures which contained only the bacillary phase *a*, germinated readily at 20° but failed to do so at 37°. Such a fact might be of considerable importance for the initiation of infection with these organisms.

It will be recalled that the different phases of the ontogenetic or pleomorphic cycle reacted somewhat differently to the Gram stain. The coccoid and fungoid phases retained the Gram stain better than did the simple non-granular bacillary phase. It is not surprising then that the phylogenetic races should exhibit a corresponding variability.

The Gram amphophilism of the individual zygospores (phase *C*) is strikingly repeated in their lineal descendant, the coccus mutant strain K. A. 105 (fig. 5). Although today it would be called a frank Gram-positive coccus it is never quite free from the Gram-negative forms, which greatly increase in number when the culture is two or three days old.

They are small and appear to arise by budding from the larger Gram-positive forms. Even in younger cultures one-half of the coccus is frequently Gram-positive and the other half Gram-negative. The ontogeny of this coccus strain reveals under suitable conditions a succession of phases beginning with large coccoid forms, tetrads, and even at times bacillary forms (fig. 5). In short the cycle is reversed from that occurring with the pure line *bacillary* culture, but the interpretation is precisely the same. This strain produces a viridans type of hemolysis and many strains of *S. viridans* are as much diphtheroid in their morphology as coccus. That this interpretation holds just as nicely for the ontogeny of the cocci as for the bacilli we have experimentally shown in Study III of this series (Mellon, 1925c).

Figure 10 shows the morphology of single cell culture of an organism in a twenty-four-hour culture on a blood agar slant, which grows in ascitic fluid or broth as figure 11. One is a diphtheroid, the other a long chained streptococcus. The literature contains numerous instances of strains of this sort of which I shall quote but one, that of Ohlmacher (1902) who showed that streptococci and *B. coli* may germinate as practically pure rods or cocci, depending on the environment. The reversibility of the phenomenon caused a negative biologic evaluation to be assigned it by bacteriologists.

Its real significance we have experimentally de nonstrated, first with the J strain (1917) and later with pure lines of the K. A. strain (1920). The actual mutation from diphtheroid to streptococcus that has been accomplished in these instances is a realization of the *potential phylogeny* represented by the ontogenetic phases of the culture. *It means, in short, that stabilization of these phases is a possibility, but only through a definite mechanism such as we have described.*

Again, the phenomenon of one phase of a culture producing acid and another phase alkali has already been set forth in Study II of this series (Mellon, 1925b). It represents one more aspect of heterogeneity that seems explained on these grounds. There is evidence, too, that alterations in virulence may often represent an aspect of a heterogeneity fundamentally no different from the others. In Study III of this series (Mellon 1925c) we have observed sudden acquisition of virulence in an alkaligenes strain that was definitely associated with a phylogenetic dissociation. Heinemann (1917) several years ago made a similar observation with the Klebs-Loeffler bacillus. This strain showed strikingly the reversible coccus and bacillary stages which could be correlated with differences in virulence. Latterly Amoss (1925) and Reimann (1925) show that pneumococci may be heterogeneous with respect to virulence, although they give no indication of the origin of that heterogeneity. It seems possible that this conception might have yielded much to the field of practical epidemiology had it not been restrained by the preconceptions to which I have referred.

FIG. 12

FIG. 13

FIG. 14

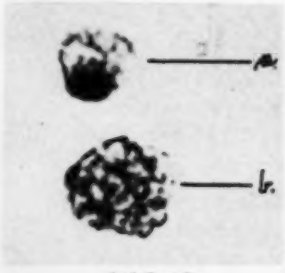

FIG. 15

FIG. 16

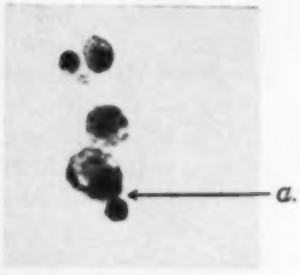

FIG. 17

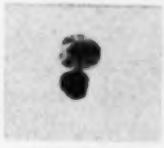

FIG. 19

FIG. 20

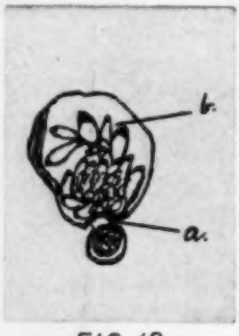

FIG. 18

FIG. 21

ATYPICAL REPRODUCTION OF B. COLI

Inasmuch as this paper has dealt with the different methods of reproduction that may occur with different phases of a single culture, it seems desirable to include here a few observations on the capacity shown by *B. coli* to bud under suitable conditions. These conditions are described in detail on page 487 of Study I of this series (1925a) and the strain under consideration is the Nx one of that paper. The forms, in addition to being larger than normal as a result of the conditions under which they were grown, were stained intravitally which avoids fixation artifacts. Moreover they were magnified from 1500 to 3000 diameters, all of which accounts for their huge size.

In figure 12 we have a bipolar organism, and in figure 13 a unipolar one with early constriction of a terminal gonidial granule. In figure 13 no division wall is present as in strict fission, while in figure 14 the snaring off of a primary bud is followed by a similar process with a secondary bud. In figure 15 are two microgonidial forms which originated from the filaments that were common with this strain. Note typical early budding at *a*, with the process more advanced in figure 16 where constriction is beginning. In figure 17 at *a* the bud is about snared off and it is particularly noteworthy that the chromatin of the maternal cell participating in the division is thrown into a skein where twenty loops can be counted in the negative. It is almost a karyokinetic evolution of the nuclear matter. Diagrammatic representation of the same is seen in figure 18 where a secondary loop formation of the chromatin is seen at *b*. Note crater formation at *a*, typical for the germinating forms of this species. In figure 19 the bud is practically freed as the daughter cell containing the deeply staining chromatin.

The method of reproduction shown in the large forms of *B. coli* (figs. 12 to 14, inclusive) can be seen in other strains of coli where the bacilli are of normal size as they grow in ordinary broth. Figures 20 and 21 at *a* show this type of reproduction which represents an intermediate stage between true fission and budding. The resulting gonidia are seen at *a* in figure 21. Although the

latter usually elongate to bacilli they may occasionally continue to grow as a diplococcoid race of *B. coli*.

This is a confirmation of the work of Hort (1917) who observed directly under the microscope similar reproductions with *B. typhosus*. Again we see that the characterization of bacteria as fission fungi must be regarded as only relatively true.

DISCUSSION

The half knowledge of these essential points bequeathed us by tradition has largely contributed to the confused and dogmatic position taken by certain sections of orthodox bacteriology in regard to them. The doctrines of monomorphism have developed no intelligent conception of the manner in which new types (developmental stages) might be *potentially present* in perfectly pure line cultures. In the past, experiments that have realized this potentiality in the dissociation of new types out of the pleomorphic cycle, have usually been prejudged on the ground that pleomorphism among the bacteria had no meaning, and the new type must have persisted *as such* in the original culture.

In recent years practical considerations in respect to the agglutination reaction leave little doubt that many bacterial cultures are heterogenous; yet so great has been the obeisance to the *involution* anachronism that it is not yet realized that the real key to the origin of this heterogeneity is inextricably associated with the "involution" forms themselves. In short, heterogeneity is the physiological counterpart of bacterial pleomorphism, and as such is a proof that the latter has important biologic connotations.

The question arises whether we shall consider as mutants the new types that may at times arise in association with sexual reorganization. To Enderlein (1925) the physiologic and cultural changes that he has shown to result from sexual reorganization in *B. cholera* constitute a new "growth generation" and cannot be considered a mutation. On theoretical grounds he inclines to the view that most of our bacterial mutants, so-called, fall in the same class. It is my opinion that these rejuvenation changes need not preclude the occurrence at times of more far reaching

ones in the same anlagen, i.e., in the gonidia or zygospore. It appears to be largely a matter of the extent of the reorganization which their environment permits to occur in them. It would be difficult to assert that the reorganization resulting in slight changes is *fundamentally* different from that producing more far reaching changes of character.

Certainly when one is able to derive organisms as different culturally, morphologically and serologically as are the strains under consideration, it would be difficult to know what to consider mutants if these are not so regarded. With no disposition to take a dogmatic view of the situation I must tentatively regard the *stabilized* stages in the phylogeny of the organisms as variants or mutants if the new characters are *constant*. If the organism gradually reverts as it ages I would then regard the "new" organism as a "growth generation."

FERMENTATION REACTIONS

The cultures were incubated for from seven to ten days at 37°C. in sugar free broth to which 1 per cent of the various sugars and bromothymol blue were added to a slight greenish tint. Good growth was obtained in all tubes. The pH of the uninoculated controls without any sugar was 7.0; of the inoculated controls 7.0 to 7.02. The readings were colorimetric except in case of doubt when they were checked with the hydrogen electrode. They were made after seven days at 37°C. and again after seven days more at 20°C.

The tubes which produced acid in from seven to ten days usually produced alkali after that period if they had not already shown it. Salicin was somewhat of an exception with the K. A. 257, inasmuch as the formation of acid was rather slow, but in fourteen days it was very definite, both by titration and colorimetrically. It will be seen that this was the only qualitative fermentation difference between K. A. 95, K. A. 191 and K. A. 257. With K. A. 105, however, mannitol, dextrin, lactose and probably insulin were fermented, which is not the case for the other three strains.

SEROLOGIC REACTIONS

Immune sera were developed in rabbits by intravenous inoculation of killed cultures of the various strains which had been isolated from a single cell, and grown on plain agar. From four to six inoculations were given over a course of two weeks. Sera were tested from seven to ten days later and if of sufficiently high titre the animals were bled and the sera put in the ice chest. Before inoculation the rabbit's sera were tested with all strains, which served as normal control sera. These were repeated when the experiments were done. The tubes were incubated two hours at 37° and read, then put in the ice chest over night and read a second time. After twenty-four hours more at 20° they were again examined.

The adsorption technique was the same as detailed in Study III of this series. K. A. 95 antiserum was developed at a time when the original bacillus was not pigmented and did not grow very luxuriantly. K. A. 191 antiserum, was made a couple of years later when the organism had acquired pigment formation, grew more luxuriantly and had lost to a large extent its capacity to form coccoids.

In the table, C indicates that agglutination is complete; 3+, 2+ and 1+ indicate a progressively lessened amount of it; − is negative; a blank space indicates no test.

Analysis of tables 2 and 3

From tables 2 and 3 it is clear that K. A. 191 and K. A. 257 agglutinate to full titre in each other's sera. Since K. A. 191 agglutinates to full titre in K. A. 95 serum it is probable that the reverse reaction would have been similar had it been possible to carry it out. On the other hand there is no evidence of cross agglutination between the coccus K. A. 105 and any of the bacillary strains, using the *usual technique.*

However, in Studies III and IV of this series (Mellon, 1925a and 1926) we have shown that it is possible at times to detect the presence of small amounts of group agglutinins by employing a temperature of 20° instead of 37° for incubation. Accordingly

TABLE 2

Agglutination reactions

ANTIGEN	TITRE OF THE HOMOLOGOUS SERA									NORMAL SERUM			NaCl 0.85	ANTISERUM
	1-20	40	80	160	320	640	1280	2500	5000	1-20	40	80		
K. A. 95	C	C	C	C	C	3+	–	–	–	–	–	–	–	K. A. 95
K. A. 257	C	C	C	C	C	C	C	3+	–	–	–	–	–	K. A. 257
K. A. 191	C	C	C	C	C	C	3+	–	–	–	–	–	–	K. A. 191
K. A. 105	C	C	C	C	3+	–	–	–	–	–	–	–	–	K. A. 105

TABLE 3

Cross agglutination reactions

ANTIGEN	1-20	40	80	160	320	640	1280	2500	5000	NORMAL SERUM			NaCl 0.85	ANTISERUM
										1-20	40	80		
K. A. 191	C	C	C	C	2+	–				–	–		–	K. A. 257
K. A. 105	1+	1+	–	–	–	–				–	–	–	–	K. A. 257
K. A. 95*													–	K. A. 257
K. A. 257	C	C	C	C	C	C	C	C	–	–	–	–	–	K. A. 191
K. A. 105	1+	1+	1+	–	–	–	–			–	–	–	–	K. A. 191
K. A. 95*													–	
K. A. 257	±1	±1	–	–	–	–	–			1+	–	–	–	K. A. 105
K. A. 191	±1	±1	±1	–	–	–	–			1+	–	–	–	K. A. 105
K. A. 95	±1	±1	–	–	–	–	–			–	–	–	–	K. A. 105
K. A. 105	−1	–	–	–	–	–	–			–	–	–	–	K. A. 95
K. A. 191	C	C	C	C	C	3+				–	–	–	–	K. A. 95
K. A. 257†													–	K. A. 95

* Not done because K. A. 95 did not regenerate when the variant K. A. 191 developed from it.

† Not done because K. A. 95 serum was exhausted at the time variant K. A. 257 was dissociated.

this was done. When K. A. 105 was run against K. A. 257 serum at 24° no agglutination could be detected, but K. A. 105 vs. K. A. 191 serum showed a definite flocculation in six tubes at the end of one hour.

Although the flocculation could be readily seen with a hand lens at the end of twenty-four hours there was little, if any, increase in it, and actual precipitation was scarcely noticeable. No agglutination occurred with the normal serum of K. A. 191 at 24°. It is desirable to employ a temperature of 20° rather than 24° as the results are usually sharper. At the time the tests were done the room temperature was 24° and it was not convenient to use 20°, especially when the adsorption test was conclusive.

Analysis of table 4

As seen in table 4 the antisera of K. A. 257 and K. A. 191 reciprocally adsorb to exhaustion the homologous agglutinins. K. A. 191 and K. A. 257 do not agglutinate in K. A. 105 serum even with the 20° technique, and the adsorption results are negative. K. A. 105 does not agglutinate in K. A. 257 serum, neither does it adsorb any agglutinins for K. A. 257 from this serum. On the other hand at 24° K. A. 105 does show partial agglutination in K. A. 191 serum and on adsorption reduces the titre of the latter from a 3+ in a dilution of 1280 (table 2) to a ±1 in a dilution of 640.

This result is of striking interest, since it appears that a pigmented diphtheroid bacillus not affecting blood agar produces group agglutinins for a frank diplococcus, non-pigmented, but producing a viridans type of hemolysis on blood agar. The sugar reactions of the two strains are also divergent. Although the reduction in titre of the serum is quantitatively not striking, it is appreciable, definite and constant for this serum.[2] The fact

[2] Several other animals were immunized with K. A.-191. These sera were adsorbed with K. A.-105 antigen and the reactions with the homologous strain varied from demonstrable ones to one that is practically inappreciable. These differences in reaction in different animals where small amounts of group agglutinin are concerned find detailed explanation in Study No. III of this series (loc. cit.).

TABLE 4

The results of adsorption

ANTIGEN	1-20	40	80	160	320	640	1280	2500	NaCl 0.85	SERUM
K. A. 257	–	–	–	–	–	–			–	K. A. 191 c Ant. 257*
K. A. 191	–	–	–	–	–	–			–	K. A. 191 c Ant. 257
K. A. 105	–	–	–	–	–	–			–	K. A. 191 c Ant. 257
K. A. 191	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 257	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 105	–	–	–	–	–	–			–	K. A. 257 c Ant. 191
K. A. 105	–	–	–	–	–	–			–	K. A. 191 c Ant. 105
K. A. 257	C	C	C	C	C	C	C	3+	–	K. A. 191 c Ant. 105
K. A. 191	C	C	C	C	C	±			–	K. A. 191 c Ant. 105
K. A. 105	–	–	–	–	–	–			–	K. A. 257 c Ant. 105
K. A. 257	C	C	C	C	C	C	C	3+	–	K. A. 257 c Ant. 105
K. A. 191	C	C	C	C	±				–	K. A. 257 c Ant. 105
K. A. 105	C	C	C	C	C	1+	–			K. A. 105 c Ant. 257
K. A. 191	–	–	–	–	–	–	–			K. A. 105 c Ant. 257
K. A. 105	C	C	C	C	C	2+	–			K. A. 105 c Ant. 191
K. A. 191	–	–	–	–	–	–	–			K. A. 105 c Ant. 191

* c stands for "adsorbed with;" Ant. for "antigen."

that there is no suggestion of such a result with strain K. A. 257 (which agglutinates and adsorbs reciprocally with K. A. 191) may mean that it is not precisely correct to base serologic identity on reciprocal adsorptions. It is probably beyond the realm of possibility to say that the antigenic complexes of two unknown strains are identical, since it would be impracticable to test out the antisera of each with a host of other strains.

The experiment is quite revealing in another direction, showing as it does that a failure of two culturally and morphologically diverse organisms to cross agglutinate does not preclude their genetic origin from a common ancestor. It is pertinent to refer to a study made by us several years ago (1919b) in which we showed that the spore and vegetative phases of the same microörganism yielded two distinct agglutinogens, from which were produced antisera that were not reciprocally adsorbed to any appreciable extent.

Strain K. A. 105 was dissociated six years ago; strain K. A. 257 was split off three years ago, and K. A. 191 four years ago. They have been quite constant in every respect since this dissociation. In order further to control this experiment an attempt was made to adsorb K. A. 191 serum with a hemolytic streptococcus (A-12) isolated at autopsy. The result was completely negative, so that we have no evidence that the cross adsorption of K. A. 105 for K. A. 191 serum could be referred to some nonspecific adsorbing factor characteristic of the streptococcus group as a whole.

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CONCLUSIONS

1. The biogenetic law of Haeckel is capable of application to the genetic history of bacteria.

2. The various phases of the pleomorphic cycle represent the ontogeny of a strain; when the phases are stabilized through mutation they collectively represent the phylogeny of this strain.

3. The bacterial classifications that *widely separate* the cocci from the bacilli do not find genetic support, irrespective of the practical value of such artificial division.

4. Group and specific agglutinogens have been demonstrated between stable races of bacilli and cocci.

5. Complete serologic reciprocity has been shown for two races of bacilli, morphologically and culturally quite diverse.

6. Considerable biologic diversity has been shown to exist among the various phases of the pleomorphic cycle. On this fact can be appropriately based the heterogeneity of pure line cultures.

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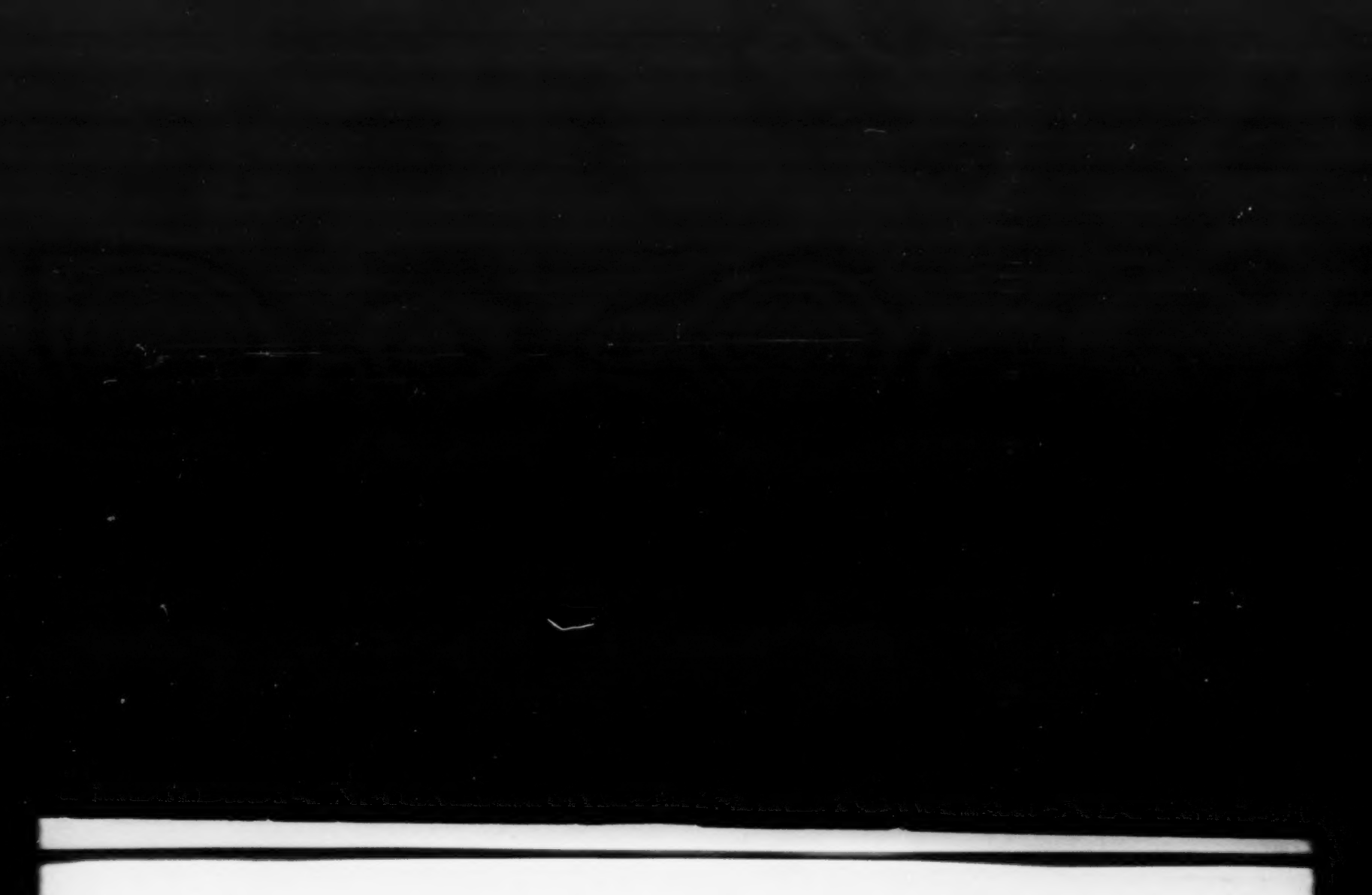
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